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Journal of Cellular Biochemistry 113:3393–3402 (2012)

Journal of Cellular Biochemistry

Proteomic Analysis of the Function of SpoT in *Helicobacter pylori* Anti-Oxidative Stress In Vitro and Colonization In Vivo

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ABSTRACT

As a microaerobe, *Helicobacter pylori* employs the global regulator SpoT for defending against oxidative stress in vitro. However, the mechanisms how SpoT affects bacterial gene expression is still unknown. Moreover, the function of SpoT in *H. pylori* colonization in the host is remaining undetermined. To explore the functions of the SpoT in *H. pylori* pathogenesis, we constructed *H. pylori* 26695 *spoT*-deficient mutant (*ΔspoT*). While grown in ambient atmosphere, protein expression profile of the *ΔspoT* was analyzed with 2D gel electrophoresis and real-time PCR. Compared to the wild type, the *spoT*-deficient strain downregulated its transcription of the oxidative-induced genes, as well as the genes responsible for protein degradation and that related to energy metabolism. Meanwhile, the colonization ability of *ΔspoT* strains in Mongolian gerbil was tested, the results demonstrated a decayed colonization in the mouse stomach with *ΔspoT* than the wild type. As a matter of facts, the AGS cells infected with the *ΔspoT* strains excreted increased level of the gastric inflammation cytokines IL-8, and the *ΔspoT* strains showed poor survival ability when treated with reactive oxygen stress (sodium nitroprusside). The elevated capacity of stimulating cytokines and fragility to reactive oxygen stress may be contribute to decreased colonization of the *spoT*-deficient mutant in the mouse stomach. Conclusively, we speculate that *spoT* is a key regulator of the genes for *H. pylori* spreading in the air and colonization in host stomach. J. Cell. Biochem. 113: 3393–3402, 2012.

KEY WORDS: *H. pylori*; OXIDATIVE STRESS; IMMUNE ESCAPE; VIRULENCE FACTORS

Helicobacter pylori is a microaerobic bacterium that colonizes the stomachs of about half of the world's population, especially in developing countries, and is associated with peptic ulceration and gastric malignancy [Blaser and Atherton, 2004]. Despite much research, the transmission and persistent colonization mechanisms of *H. pylori* remain poorly understood.

Nearly all living microorganisms, especially anaerobes and microaerobes, must cope with oxidative stress. *H. pylori* is exposed to ambient atmosphere during the person-to-person transmission process, and hence exposure to oxygen and oxidative stress injury is unavoidable. Although an oxygen tension of 20% in the atmosphere is not suitable for the long-term survival of this bacterium, it is

The authors have no competing interests.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81071313, 30800037, 81171536, 30972775, 30971151; Grant sponsor: The Science Foundation of Shandong Province, China; Grant numbers: ZR2009CZ001, ZR2009CM002, BS2010YY038, BS2010YY040, BS2011SW023; Grant sponsor: The National Basic Research Program of China; Grant number: 2012CB911202.

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Manuscript Received: 29 March 2012; Manuscript Accepted: 1 June 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 June 2012

DOI 10.1002/jcb.24215 • © 2012 Wiley Periodicals, Inc.

sufficient for successful transmission between hosts. Thus, *H. pylori* must have a protective mechanism to defend itself against short-term oxygen shock but not long-term oxidative stress. Previous studies have reported that *H. pylori* reduces oxidative stress and toxic reactive peroxide radical levels by upregulating the expression of alkylhydroperoxide reductase (AhpC/TsaA), neutrophil activating protein (NapA), catalase, and NADPH quinone reductase [Olczak et al., 2002; Comtois et al., 2003]. The regulatory mechanisms are unclear, although the ferric uptake regulator Fur [Ernst et al., 2005; Alamuri et al., 2006] and the post-transcriptional regulator *CsrA* have been implicated [Barnard et al., 2004].

Persistent colonization by *H. pylori* will induce an innate and adaptive immunity response. The innate immune system involves pattern-recognition receptors to recognize the invasive microbiology such as toll-like receptors (TLRs), which signal through MyD88 or TRIF to initiate production of proinflammatory cytokines [Casanova et al., 2011]. *H. pylori* can induce TLR-2 and TLR-5 activation, thus leading to the secretion of the potent neutrophil chemoattractant interleukin 8 (IL-8) [Kumar Pachathundikandi et al., 2011]. It is particularly worth mentioning is CagA, an important virulence factor of *H. pylori,* can enhance the production of IL-8 in human stomach [Peek et al., 1995; Kim et al., 2006], cooperating with NapA to recruit human neutrophils and monocytes to the site of infection [Brisslert et al., 2005]. As well, *H. pylori* urease can also recruit phagocytes to the lamina propria [Allen, 2000], and ultrastructural analysis of biopsy samples has revealed large numbers of neutrophils traversing the gastric epithelium and encountering *H. pylori* in the mucus layer [Zu et al., 2000]. With the aid of its virulence genes that participates in megasome formation, such as urease and possibly *cag*-PAI, *H. pylori* evades phagocytosis and defend against oxidative stress from neutrophils and monocytes contributing to *H. pylori* survival and long-term colonization in the gastric mucosal [Allen, 2000]. However the regulatory mechanism for *H. pylori* persistent colonization is rarely reported.

The stringent response is bacterial adaptation that occurs during nutrient limitation and under other stressful conditions. It involves the global variation of transcription characterized by the repression of ribosomal genes and the activation of specific stress response genes [Kvint et al., 2000]. In *Eschericia coli*, the stringent response is regulated by *relA* and *spoT*, which are synthetases for guanosine tetra- and penta-phosphate [(p)ppGpp] [Cashel et al., 1996; Durfee et al., 2008]. Previous research suggested that these enzymes were essential for several bacterial pathogens during infection and transmission (Mittenhuber, 2001; Gaynor et al., 2005; Braeken et al., 2006). Differing from *E. coli*, the *H. pylori* genome contains only *spoT* and lacks *relA*. It also lacks sigma *S*, *H*, and *E*, which are typically involved in the stringent responses in many gram-negative bacteria [Alm et al., 1999]. Thus, *spoT* may be a critical regulator of stress responses. As a matter of proof, recent research demonstrated that *spoT* is required for *H. pylori* survival in the stationary phase under the circumstance of exposure to acid, and aerobic shock, as well as on nutrient and pH downshift [Mouery et al., 2006; Wells and Gaynor, 2006]. SpoT can also regulate the growth of *H. pylori* during serum starvation and bacterial survival in macrophages [Zhou et al., 2008].

The mechanism by which *spoT* contributes to *H. pylori* defense against oxidative stress and persistent colonization remains unclear. Here we compared the protein expression profiles of *H. pylori* 26695 *spoT* deficient (*ΔspoT*) strains with wild-type by 2D electrophoresis (2-DE) after exposure to ambient atmosphere. Additionally, their difference in IL-8 induction and resistance to reactive oxygen stress as well as concerning ability to colonize in the host were interrogated at the same time.

METHODS

CONSTRUCTION OF *H. pylori* 26695 *spoT*-DEFICIENT MUTANT (*ΔspoT*)

Plasmids pILL570 and pUC18K2 were kindly provided by Professor Agnès Labigne (Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur). The *spoT* mutant was constructed as described [Qu et al., 2009]. The genome of *H. pylori* 26695 was extracted as a template. The primers are listed in Table I.

STRAINS AND CULTURE CONDITIONS

H. pylori 26695 was kindly provided by Dr. Zhang Jianzhong (Chinese Disease Control and Prevention Center). Wild-type and *ΔspoT H. pylori* strains were grown as described [Chuang et al., 2005]. The *spoT*-deficient strain was grown on Skirrow agar (SA) plates with 5% (v/v) sheep blood, supplemented with 10 μg kanamycin (Sigma–Aldrich, St. Louis, MO). SA plates were incubated at 37°C and 10% CO_2. Liquid culture media for *H. pylori* consisted of *Brucella* broth containing 10% fetal bovine serum for incubation in a microaerobic environment (5% O_2, 10% CO_2, and 85% N_2) at 37°C on a shaker set at 120 rpm.

PREPARATION OF *H. pylori* PROTEINS FOR 2-DE

The wild-type and *ΔspoT H. pylori* grown on SA plates were exposed to atmosphere for 2 h, then harvested and resuspended in lysis buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, 1% pharmalyte (pH range 3–10), 1% protease inhibitor, and 1% nuclease mix (Amersham Biosciences, GE Healthcare, Shanghai). After sonication, the solution was centrifuged at 20,000*g* for 60 min at 4°C, and the precipitate was discarded. The Bradford method was used to determine protein concentration and approximately 250–300 mg protein was loaded onto an 18-cm immobilized pH gradient (IPG) strip (pH 3–10 NL). Isoelectric focusing involved use of an IPGphor instrument (Amersham Biosciences). Proteins in the IPG strips underwent 12% SDS–PAGE. The gels were silver stained and scanned by use of an ImageScanner II (Amersham Biosciences). 2-DE was repeated three times with independently grown cultures. Image analysis involved ImageMaster 2D Elite v5.0 (Amersham Biosciences). A two-fold change ($P < 0.05$) in protein spot volume was defined as significant.

IN-GEL DIGESTION AND MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT (MALDI-TOF) MASS SPECTROMETRY (MALDI-TOF/TOF MS)

To perform 2-DE gel analysis, significantly changed protein spots were excised and digested with trypsin. By use of a C18 ZipTip

TABLE I. Primers Used in This Study

Primers	Sequence (5′–3′)	Primers	Sequence (5′–3′)
spoT-1	CCATCGATGGCGAGCCTTATATTGTCCATC	*spoT*-2	CGGAATTCTTCCAGTGGGCTGAATTACC
spoT-3	CGGGATCCGCGATCGCTTTAGATTTTGC	*spoT*-4	AACTGCAGCTCTGATCCAATCCGCCTTATC
16S rRNA F	GCGTCATCACCAATAAGCC	*porD* F	GCCATTACACCGAGCAAA
16S rRNA R	GACAGCCATTTGTGCGAGA	*porD* R	GGGCAATAAACCCAACAA
spoT F	AAGTGCAGATCCGCACCTTT	*clpP* F	GTGGCGTCTTCTATCGTG
spoT R	GCAACCATCTCATGCCCTCA	*clpP* R	AATCGTGGAAACATCAGG
fldA F	CTAGAAGCGAGCGATTTTGC	*clpX* F	CTAATCGGCCCTACAGGA
fldA R	AACCATCAGTGGGAGTTTGC	*clpX* R	CACATAGCCCGCTTCAGT
sodB F	AATCTCATCAAAGGCACG	*tagD* F	ATCGGTTTGTTTGCTCCAAG
sodB R	GCTTAGGCAATCCCAATA	*tagD* R	AACAGCACGCCGTAATTTTC
ureC F	GGCTAGTGGTGGTGGATA	*porB* F	AAAATCACTTACCGCCCTAG
ureC R	GACAACCGCTTGAGAAGA	*porB* R	TCCCAACGCTCATTCACA
napA F	TGAAGAGTTTGCGGACAT	*tsaA* F	GAAAAAGGCGGTATTGGTCA
*nap*A R	AGAGTGGAAGCTCGTTTT	*tsaA* R	GATCACCGCATGCCTTACTT
*cag*A F	AGCAAAAAGCGACCTTGAA	*kata* F	CGTTTGGGATGACAACAA
*cag*A R	AGCCAATTGCTCCTTTGAGA	*kata* R	TAAGCTCCGCTTCCTTTA
GPADH F	AACTTTGGCATTGTGGAAGG	GPADH R	ACACATTGGGGGTAGGAACA

F: forward primer; R: reversed primer.
Shading indicates nucleotides that were added at 5′ end to create a restriction site.
Restriction sites for *Cla*I (spot-1), *Eco*RI (spot-2), *Bam*HI (spot-3), and *Pst*I (spot-4) are underlined.

(Millipore, Billerica, MA), the tryptic digests were desalted, then mixed with α-cyano-4-hydroxycinnamic acid, and spotted onto MALDI target plates. Peptide mass fingerprints were obtained on a 4700 MALDI-TOF/TOF tandem mass spectrometer (Applied Biosystems). The MS and MS/MS spectra were analyzed with 50 ppm mass tolerance by use of GPS Explorer v2.0.1 and Mascot v1.9 based on NCBI SWISS-PROT and local *H. pylori* databases (updated April 2006). Background peaks from known trypsin autodigestion fragments and common contaminants were excluded before searching the databases. Oxidation of methionine and carbamidomethylation of cysteine were allowed as variable modifications. Identification with a GPS confidence interval >99% was accepted.

ANALYSIS OF COLONIZATION OF WILD-TYPE AND *ΔspoT H. pylori* INFECTION IN MICE

Mongolian gerbils (*Meriones unguiculatus*) were purchased from ZheJiang University and bred at the Shandong University pathogen-free facility. Gerbils were housed in individually ventilated cages and at 7–8 weeks of age were divided into two groups (n = 5) for infection with wild-type and *ΔspoT*. All animal experimental protocols were as described [Ohkusa et al., 2003] and were in accordance with institutional guidelines. The kinetics of colonization in mice with *H. pylori* 26695 infection have been well described, showing stable colonization between 4 and 8 weeks of infection [Ohkusa et al., 2003]. To determine the kinetics of colonization with wild-type and *ΔspoT H. pylori* infection in mice, animals were killed at various times after infection (weeks 4 [n = 5], 6 [n = 5], and 8 [n = 5]). Stomachs were removed, then the antrum was cut and washed with phosphate-buffered saline (PBS) to eliminate food residue. Antrum homogenates from *ΔspoT*-infected mice were cultured on blood SA plates with kanamycin. The mucus layer was removed from the antrum on a glass slide and placed in extraction buffer (20 mM Tris, pH 8.0, 0.5% Tween 20, 0.5 mg/ml proteinase K), incubated for 1 h at 55°C, then 10 min at 98°C, then centrifuged for 5 min at maximum speed. The supernatants were stored at −20°C before analysis by real-time PCR with *H. pylori ureC* gene as described [He et al., 2002]. Primers for *ureC* are in Table I.

WILD-TYPE AND *ΔspoT H. pylori* INFECTION IN VIVO IN C57BL/6 MICE

For *spoT* mutant strains can not be effective colonization in mice, virulence gene expression differences between wild and mutant strains only be done in the initial stages of infection, so we infected C57BL/6 mice in short-time used wild and *ΔspoT H. pylori*, C57BL/6 mice were bred at the Shandong University pathogen-free facility. Mice were randomly divided into two groups for treatment (n = 5). The mice fasted for 2 days to ensure gastric emptying. Mice received approximately 10^{10} CFU ml^{-1} wild-type or *ΔspoT H. pylori* suspension in a 0.5-ml volume by use of oral gavage for 4 h, then mouse stomachs were opened, washed in PBS to collect the bacteria. Total RNA was extracted from bacteria and mRNA expression was measured by real-time RT-PCR.

QUANTITATIVE (REAL-TIME) RT-PCR

Total RNA was isolated by use of TRIzol reagent (Invitrogen, Carlsbad, CA). After the DNase I process, cDNA was obtained by use of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, MD). The primers are in Table I. In total, 10 μl SYBR Premic Ex TaqTM (Takara, Otsu, Shiga, Japan) and 0.4 μl ROX Reference Dye (Takara) were added to each 20-μl PCR reaction mixture. Real-time RT-PCR involved the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA) for one cycle at 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 31 s. Melting curve analysis for each PCR reaction was performed to ensure the purity of the amplified product. The data were normalized to 16s RNA expression in each sample and three biological replicates were performed. Primers are in Table I.

IL-8 ELISA

Human AGS gastric cancer cells were cultured in F12 medium (Gibco Life Technologies) with 10% fetal bovine serum (FBS; Gibco Life Technologies), after co-culture in quadruplicate with wild-type and *ΔspoT H. pylori* for 24 h (37°C) in 95% air, 5% CO_2 humidified air. Supernatants were aspirated at 24 h and stored at −80°C. IL-8 secreted by AGS cells stimulated with *H. pylori* was detected by use

of the Human IL-8/NAP-1 Platinum ELISA Kit (eBioscience, San Diego, CA).

SURVIVAL ABILITY OF WILD-TYPE AND *ΔspoT H. pylori* EXPOSED TO SODIUM NITROPRUSSIDE (SNP)

This experiment was according to our previous method [Qu et al., 2009]. Aliquots (20 ml) of cell cultures were added with SNP (4 mM) in flasks under microaerobic conditions at 37°C. At 3-h intervals, the OD_{600} value was measured, a 10-μl suspension was applied to an SA plate with 5% (v/v) sheep blood under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C and incubated for 3–4 days before viability assessment.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Statistical significance was determined by unpaired Student's *t* test and one-way ANOVA. $P < 0.05$ was considered statistically significant. Results were analyzed using GraphPad Prism (GraphPad Software Inc., CA).

RESULTS

MOST OF THE GENES IN *ΔspoT H. pylori* CULTURED IN AMBIENT ATMOSPHERE WERE DOWNREGULATED ON PROTEIN AND mRNA LEVEL

We compared the protein expression profiles in *ΔspoT* and wild-type *H. pylori* exposed to ambient atmosphere in digital images of 2-DE gels (Fig. 1). In total, 51 protein spots were differentially transcribed ($P < 0.05$) in *ΔspoT* (Table II; Fig. 1). The main spots showing differential regulation represented proteins involved in energy metabolism (Fig. 2A), antioxidation (Fig. 2B), and protein fate (Fig. 2C), respectively. The results were further confirmed in mRNA levels by RT-PCR, which demonstrated a similar down-regulation in *ΔspoT H. pylori* (Fig. 2).

COLONIZATION ABILITY OF *ΔspoT H. pylori* WEAKENED IN GASTRIC SYSTEM OF MONGOLIA GERBILS

In order to study if *SpoT* contributes to *H. pylori* colonization in mice, we compared the survival ability of wild and *ΔspoT* mutant strains, the colonization ability of *ΔspoT* was weaker than the wild type in the gastric system of Mongolia gerbil (Fig. 3).

ΔspoT H. pylori INFECTION INDUCED MORE IL-8 SECRETION IN VITRO

IL-8 is one of the important factors to induce a strong immune response, it has been known that *H. pylori* infection can induce more IL-8 secretion in AGS cells [Sharma et al., 1995] and patients [Ando et al., 1996], so we checked the IL-8 secretion level form AGS cells infected by wild and mutant strain with ELISA, the result showed AGS cells infected by *ΔspoT H. pylori* secreted elevated amount of IL-8 than the wild-type within 24 h (Fig. 4A).

ΔspoT H. pylori INFECTION INCREASED mRNA LEVELS OF NapA AND CagA IN VIVO IN MICE

NapA and CagA are important virulence factor of *H. pylori*, both of them can induce host immunity response to affect the bacteria colonization, so we analyzed their expression differences at the time of infection. Bacteria isolated from the stomach antrum of C57BL/6 mice with short-term infection showed higher expression of CagA and NapA concomitant with *spoT* deficiency (Fig. 4B).

SURVIVAL OF *ΔspoT H. pylori* WAS WEAKENED WITH SNP EXPOSURE

As one of the active oxygen species, NO has protective roles in the acute and persistent phases of *H. pylori* infection. We used SNP (sodium nitroprusside) as an NO donor to simulate the antimicrobial process of NO in *H. pylori* in vitro. The results showed lower survival rate with *ΔspoT H. pylori* than that of the wild type (Fig. 5).

DISCUSSION

SpoT TAKES PART IN *H. pylori* RESISTING OXIDATIVE STRESS IN VITRO

Aerobic environment is inevitable for *H. pylori* to face in the process of transmitting from the present host to a new host, and the Δ*spoT* mutant showed impaired survival ability under aerobic stress [Mouery et al., 2006], so we deduced that SpoT played crucial roles in anti-oxidative stress. However, the mechanism remains unexplored. Thus we analyzed the expression profiles of oxidative stress proteins in *ΔspoT* and wild-type *H. pylori* grown on SA plates and exposed to the atmosphere which was typically an oxidative condition. As expected, the density of most spots in 2D gel map were reduced, including those proteins involved in amino acid biosynthesis, protein synthesis, redox reaction, protein fate, as well as the transcription termination factor NusG, which may explain the low involvement in protein synthesis and amino acid biosynthesis (Fig. 1B,C). However, the protein expression differences between the wild-type and Δ*spoT* were mainly in proteins involved in energy metabolism, detoxification, and protein fate (Fig. 1B,C; Table II).

The oxidative decarboxylation of pyruvate is an important reaction in bacteria. For some aerobic bacteria, this reaction is catalyzed by the pyruvate dehydrogenase multi-enzyme complex [Patel and Roche, 1990]. The bacteria are capable of undergoing mixed acid fermentation under anaerobic conditions. The generation of acetyl-CoA from pyruvate is catalyzed by pyruvate:formate lyase; however, biochemical and genomic data have revealed both of these enzymes absent in *H. pylori*, and instead this reaction is catalyzed by an unusual 4-subunit pyruvate:flavodoxin oxidoreductase (POR) [Hughes et al., 1995, 1998]. Commonly, the reduction of low-potential electron acceptors is catalyzed by three enzymes containing ferredoxin or flavodoxin proteins in vivo. The electron acceptor of the *H. pylori* POR enzyme is a flavodoxin (FldA; HP1161) in vivo [Hughes et al., 1995, 1998]. *H. pylori* POR is highly oxygen labile, which may be critical for *H. pylori* survival in an microaerophilic condition [Hughes et al., 1995; Kelly, 1998]. Mutant of the *porB* gene is not viable, implying that POR is essential for the growth of *H. pylori* [Hughes et al., 1995]. Our proteomic analysis revealed that *porB*, *porD* and *fldA* were significantly decreased in the *spoT* mutant versus wild-type control (Fig. 2A), so the pyruvate metabolism of *ΔspoT* was reduced, which would affect

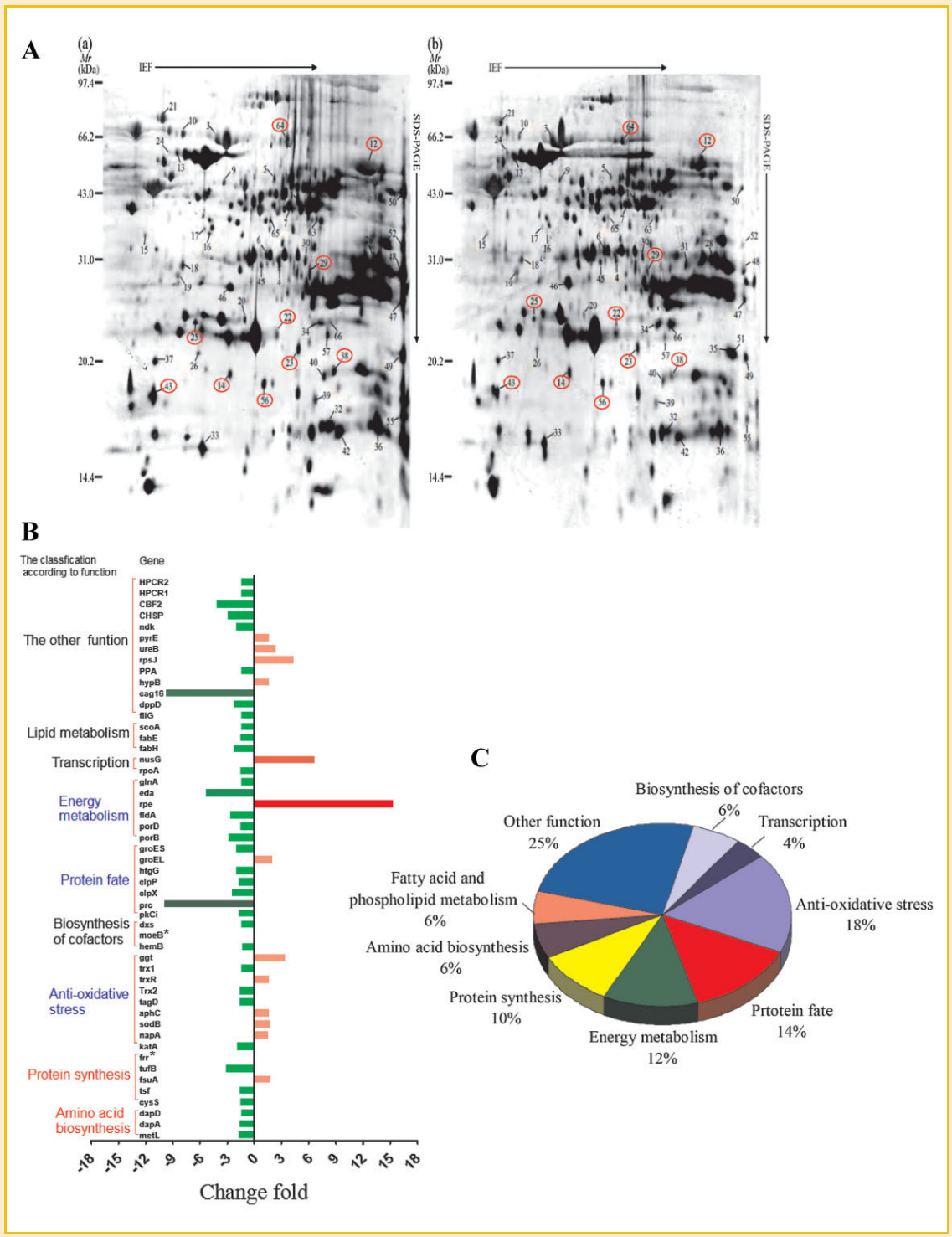

Fig. 1. Proteomics analysis of differentially expressed proteins between wild-type and *ΔspoT* mutant *Helicobacter pylori*. A: Representative 2-DE maps of wild type (a) and *spoT*-deficient strains (b) exposed to atmosphere for 2 h. IEF, isoelectric focusing. Expression of spots with red circles were comfirmed by real-time PCR. B: Cluster analysis. Expression of proteins in *ΔspoT*; upregulated proteins are in red, and downregulated proteins are in green. The intensity of the green or red color corresponds to the degree of regulation as indicated by number at the bottom of the figure. The main proteins involved in anti-oxidative stress are marked in red and blue; *present only in *ΔspoT*; HPCR: *H. pylori* hypothetical protein, CBF2: HP1285, CHSP: HP0175. C: Gene ontology classification of the 51 proteins with differential expression: anti-oxidative stress (18%), protein fate (14%), energy metabolism (12%), protein synthesis (10%), amino acid biosynthesis (6%), fatty acid and phospholipid metabolism (6%), biosynthesis of cofactors (6%), transcription (4%), and other function (25%).

TABLE II. Analysis of Proteomics Involved in Anti-Oxidative Stress Between *H. pylori* 26695 and *H. pylori* (*ΔspoT*) Exposed to the Atmosphere 2 h

The classfication according to function		Spot no.[a]	TIGR ORF no.[b]	Protein name	Gene	Change fold[c]
Amino acid biosynthesis	Aspartate family	1	HP0822	Homoserine dehydrogenase	metL	−1.688
		30	HP1013	Dihydrodipicolinate synthetase	dapA	−1.564
		63	HP0626	Tetrahydrodipicolinate N-succinyltransferase	dapD	−1.389
Protein synthesis	tRNA aminoa-cylation	5	HP0886	Cysteinyl-tRNA synthetase	cysS	−1.480
	Translation factors	4	HP1555	Translation elongation factor EF-Ts	tsf	−1.593
		24	HP1195	Translation elongation factor EF-G	fusA	+1.828
		65	HP1205	Translation elongation factor EF-Tu	tufB	−3.096
		35	HP1256	Ribosome releasing factor	frr	Present
Anti-oxidative stress	Detoxification	12	HP0875	Catalase	katA	−1.817
		14	HP0243	Neutrophil activating protein	napA	+1.584
		22	HP0389	Superoxide dismutase	sodB	+1.716
		23	HP1563	Alkyl hydroperoxide reductase	aphC/tsaA	+1.624
		38	HP0390	Adhesin-thiol peroxidase	tagD	−1.548
	Thioredoxin, glutaredoxin, and glutathione	42	HP1458	Thioredoxin	trx2	−1.536
		45	HP0825	Thioredoxin reductase	trxR	+1.618
		33	HP0824	Thioredoxin	trx1	−1.364
		48	HP1118	Gamma-glutamyltranspeptidase	ggt	+3.398
Biosynthesis of cofactors		21	HP0163	Delta-aminolevulinic acid dehydratase	hemB	−1.316
		51	HP0814	Molybdopterin biosynthesis protein	moeB	Present
		7	HP0354	Deoxyxylulose-5-phosphate synthase, putative	dxs	−1.386
Protein fate	Degradation of proteins, peptides, and glycopeptides	39	HP0404	Protein kinase C inhibitor		−1.712
		50	HP1350	Protease	prc	−9.911
		64	HP1374	ATP-dependent protease ATPase subunit	clpX	−2.482
		25	HP0794	ATP-dependent clp protease proteolytic component	clpP	−1.678
	Protein folding and stabilization	10	HP0210	Chaperone and heat shock protein C62.5	htpG	−1.925
		13	HP0010	Chaperone and heat shock protein	groEL	+1.976
		32	HP0011	Co-chaperone	groES	−1.743
Energy metabolism	Anaerobic	29	HP1111	Pyruvate ferredoxin oxidoreductase, beta subunit	porB	−2.841
		56	HP1109	Pyruvate ferredoxin oxidoreductase, delta subunit	porD	−1.256
	Electron transport	43	HP1161	Flavodoxin	fldA	−2.660
	Pentose phosphate pathway	66	HP1386	D-ribulose-5-phosphate 3 epimerase	rpe	+15.328
	Entner-Doudoroff	31	HP1099	2-keto-3-deoxy-6-phosphogluconate aldolase	eda	−5.255
	Nitrogen metabolism	9	HP0512	Glutamine synthetase	glnA	−1.404
Transcription	DNA-dependent RNA polymerase	15	HP1293	DNA-directed RNA polymerase, alpha subunit	rpoA	−1.478
	Transcription factors	57	HP1203	Transcription termination factor NusG	nusG	+6.618
Fatty acid and phospholipid metabolism		16	HP0202	Beta-ketoacyl-acyl carrier protein synthase III	fabH	−2.270
		26	HP0371	Biotin carboxyl carrier protein	fabE	−1.506
		20	HP0691	3-oxoadipate coA-transferase subunit A	scoA	−1.382
Purines, pyrimidines, nucleosides, and nucleotides: pyrimidine ribonucleotide biosynthesis		34	HP1257	Orotate phosphoribosyltransferase	pyrE	+1.645
		36	HP0198	Nucleoside diphosphate kinase	ndk	−1.938
Secreted protein		47	HP1285	Conserved hypothetical secreted protein		−2.910
		49	HP0175	Cell binding factor 2		−4.122
Hypothetical protein		17	HP0049	*H. pylori* predicted coding region		−1.400
		55	HP0614	*H. pylori* predicted coding region		−1.435
Phosphorus compounds		37	HP0620	Inorganic pyrophosphatase	PPA	−1.360
Cellular processes: chemotaxis and motility		18	HP0352	Flagellar motor switch protein	fliG	−1.365
Ribosomal proteins, synthesis and modification		40	HP1320	Ribosomal protein S10	rpsJ	+4.407
Urea		3	HP0072	Urease subunit bete	ureB	+2.366
Transport and binding proteins: amino acids, peptides, and amines		28	HP0301	Dipeptide ABC transporter, ATP-binding protein	dppD	−2.095
Cellular processes: pathogenesis		52	HP0537	Cag pathogenicity island protein	cag16	−9.709
Electron transport		46	HP0900	Hydrogenase expression/formation protein	hypB	+1.593

[a]Spot numbers refer to the proteins in Figure 2.
[b]TIGR ORF no. follows the nomenclature of *H. pylori* strain26695.
[c]Change fold for each protein derived from *spoT* mutant compared with the protein derived from wild-type *H. pylori* 26695; Present: the protein only presents in *spoT* mutant.

the tricarboxylic cycle process and consequently weakened associated biosynthesis and catabolism, leading to poor survival of the *ΔspoT* strains while exposed to the atmosphere.

ΔspoT is susceptible to oxidative stress [Mouery et al., 2006]. Our 2-DE maps (Fig. 1B; Table II) showed that several proteins involved in antioxidative stress were differently expressed. The expression levels of *sodB*, *aphC/tsaA*, *trxR* and *napA* were upregulated and those of *tagD*, *katA*, *trx1*, and *trx2* downregulated (Fig. 2B; Table II). According to the complete system catalyzing the reduction of peroxides (ROOH) [Baker et al., 2001], catalysis reduction of NADPH to ROOH is by TrxR, Trx1, and AphC/TsaA. Exposing *ΔspoT H. pylori* to atmosphere resulted in increased TrxR and AphC/TsaA expression, with decreased Trx1 expression (Table II). Reduced production of Trx1 trigerred defective ROOH synthesis. On the other hand, most ahpC/tsaA mutants, the expression of NapA is abnormally high [Olczak et al., 2002, 2005], in our study the protein expression level of NapA was also high, which may be a compensatory mechanism for the low activity of ROOH, but its relative mRNA expression was reduced, as well as SodB (Fig. 2B).

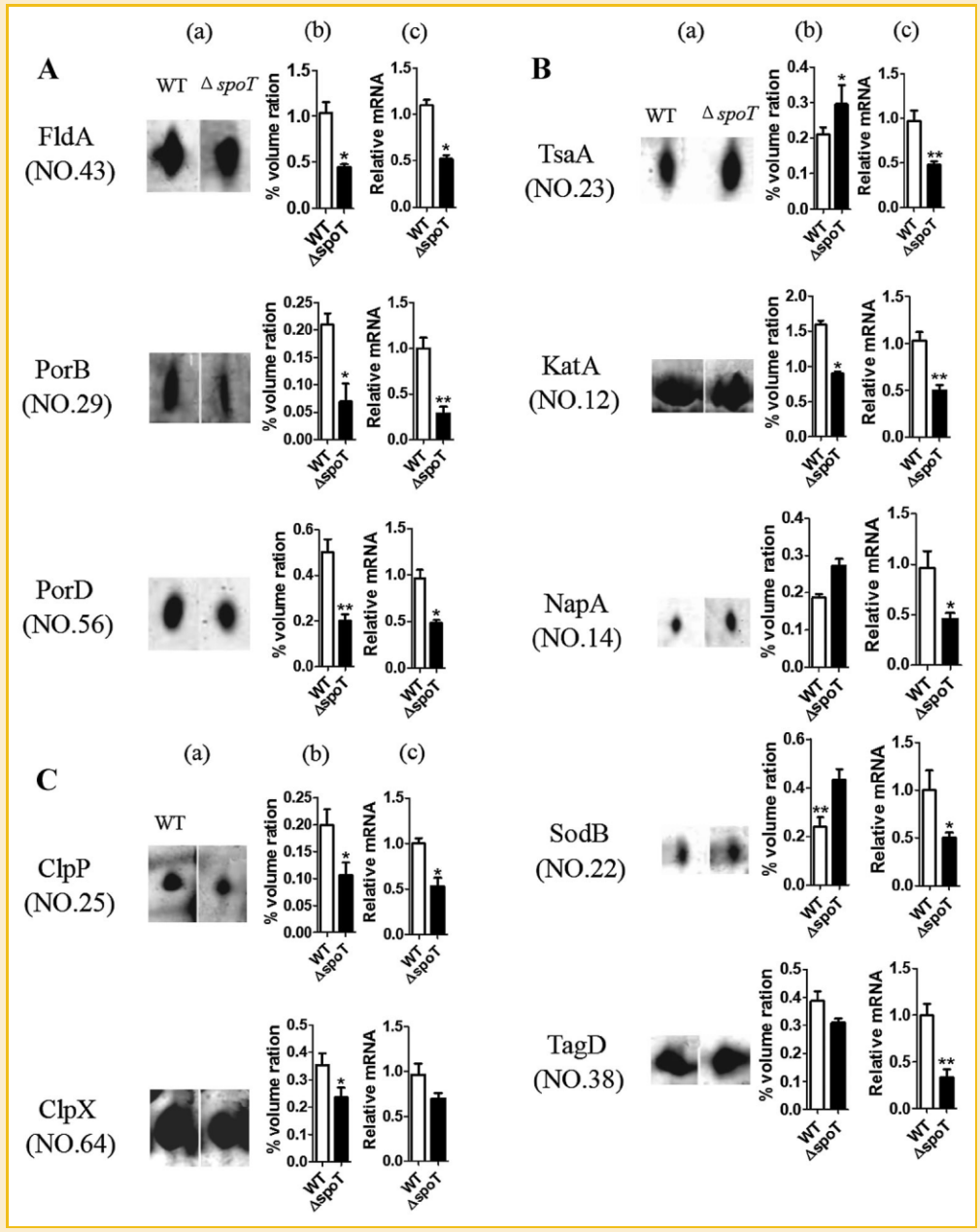

Fig. 2. The protein and mRNA expression of most factors was lower in *ΔspoT* than the wild type (WT) exposed to atmosphere. A: mRNA and protein levels of WT and *ΔspoT* involved in energy metabolism; (B) Antioxidation and (C) protein fate mRNA and protein levels. a: Total protein spot volume ratio from 2-DE analysis; (b) Quantification of total protein spot volume ratio from 2-DE analysis; (c) mRNA levels assessed by real-time PCR; signals were normalized to 16s rRNA levels. Data are mean ± SD of replicated experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Another genes, Trx2 and TagD, were also downregulated in *ΔspoT* strain(Figs 1 and 2B), yet their function are also still not clear. Trx2 mutants were found more sensitive to oxidative and nitrosative stress than the wild type [Comtois et al., 2003]. TagD belongs to peroxiredoxins, which have thioredoxin-linked peroxidase activity. The *H. pylori* tagD mutant showed more sensitivity to peroxide and superoxide than the wild type [Comtois et al., 2003]. Trx2 and TagD may contribute to the poor survivability of *ΔspoT* strains in vitro.

Catalase catalyzes the degradation of H_2O_2 into water and oxygen to protect cells against the damaging effects of H_2O_2 [Nicholls et al., 2001]. Previous study reported that the viability of the *katA* mutant was poor after exposure to 10 mM H_2O_2 as compared with the wild type [Harris et al., 2002, 2003]. Our research revealed that the expression of *katA* was also reduced in *ΔspoT* under ambient atmosphere (Fig. 2B), which may be associated with decreased survival of *H. pylori*.

Existing elevated levels of damaged or misfolded proteins may not only owing to the increasing rate of errors in transcription or translation, but also may be owing to the stringent environments (e.g., the action of excessive heat, acid or reactive oxygen species). A

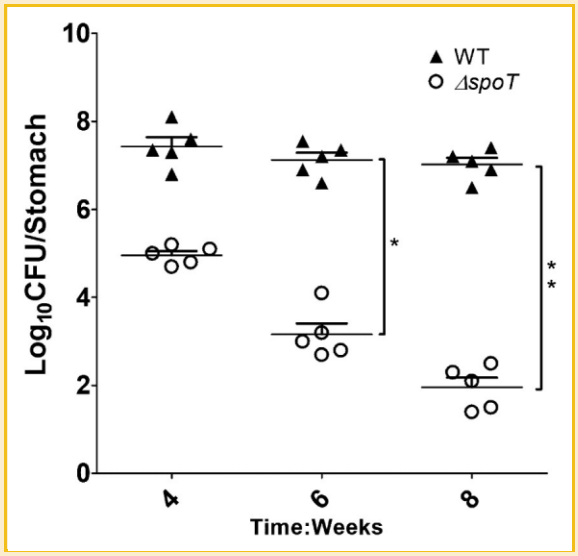

Fig. 3. The Δ*spoT* mutant shows a defect in colonization in gastric system of Mongolia gerbils (n = 5/group). $^{*}P < 0.05$, $^{**}P < 0.01$. CFU = colony formation unit.

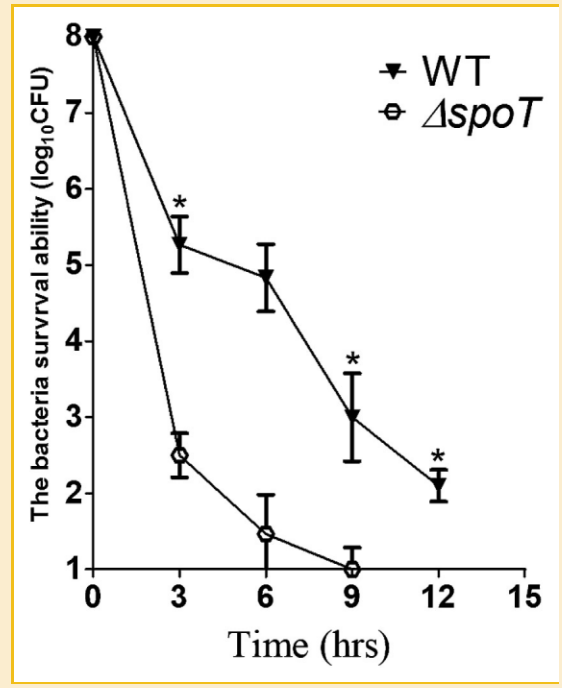

Fig. 5. Δ*spoT* mutant has a survival defect on exposure to sodium nitroprusside. WT and *ΔspoT* were grown by shaking under microaerophilic conditions at 37°C, and the number of colony formation units (CFU) ml^{-1} were determined at the times indicated. A representative assay of at least three trials is shown. Data are mean ± SD of replicated experiments. The limits of detection of the assays were 10 CFU ml^{-1}. $^{*}P < 0.05$.

number of bacterial proteases and associated molecules that assist in turnover of proteins have been reviewed (Gottesman, 1996; Butler et al., 2006). These proteases and chaperones are important in assuring the quality of protein synthesis both in normal and stressful environments. Among them, both ClpX and ClpP function as components of proteases and were ATP dependent [Wickner et al., 1999]. Besides assisting in protein folding as chaperones, ClpX plays an important role in metabolism [Loughlin et al., 2009], but c*lpX* deletion mutants are not viable to further study the function of this protein [Robertson et al., 2003]. It had been proved that *H. pylori* SS1 strains with inactivated *clpA* or *clpP* genes showed slightly increased sensitivity to oxidative stress from H_2O_2, cumene hydroperoxide or methyl viologen, whereas the double mutant demonstrated a much greater sensitivity [Robertson et al., 2003]. In our study, the ClpP and ClpX are down-regulated in *H. pylori* 26695 *ΔspoT* strain while exposed to the atmosphere (Figs 1B and 2C), indicating ClpX and ClpP may take part in the reduced viability of *H. pylori* 26695 (*ΔspoT*).

SPOT MAY INVOLVE IN IMMUNE ESCAPE OF *H. PYLORI* IN VIVO

In our study, we found that the number of the mice successfully colonized by *spoT* mutant in stomach was much less than the wild

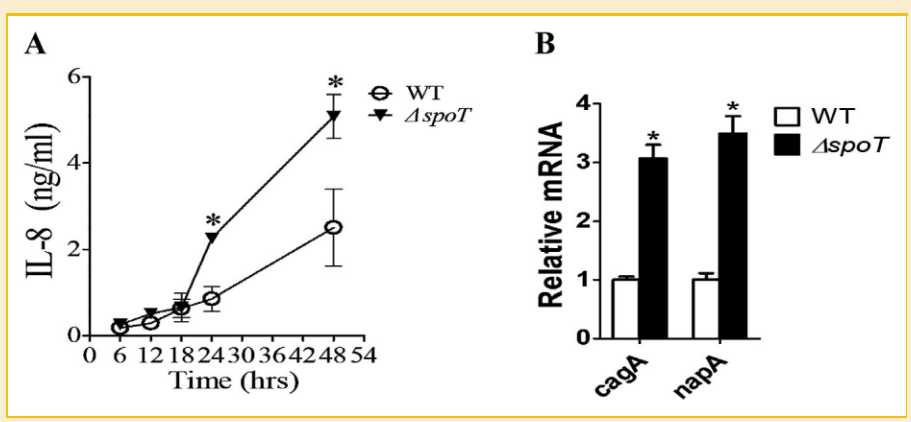

Fig. 4. *ΔspoT* induces more inflammation than the wild type *H. pylori* in vitro and in vivo. A: IL-8 concentration in co-culture supernatants of WT and *ΔspoT* and AGS cells by ELISA. B: mRNA expression of *napA* and *cagA* in WT and *ΔspoT* with short-term(4 h) infection in C57BL/6 mice. Data are mean ± SD of replicated experiment. $^{*}P < 0.05$.

strain (Fig. 3). The weak resistance of *spoT* mutant to acid [Mouery et al., 2006] and phagocytosis by macrophages [Zhou et al., 2008] may contribute to this bad survival ability. However, our data indicated that a stronger IL-8 (Fig. 4A) response was stimulated by *spoT* mutant and the sensitivity to NO (Fig. 5) may also be another reason responsible for its weak colonization ability. *H. pylori* 26695 *ΔspoT* strain expressed higher level of *cagA* and *napA* mRNA (Fig. 4B), which may be responsible for the higher induction of IL-8 and NO.

H. pylori can persistently colonize in gastric mucosa unless cleared by antibiotics, implying that it has an effective strategy to evade the host immune response. A good example is *H. pylori* LPS, it has special structure and is able to escape the recognition by host TLR4 [Ferrero, 2005] compared with LPS from *Escherichia coli* or *Salmonella typhimurium* [Muotiala et al., 1992; Bliss et al., 1998]. Similarly, *H. pylori* flagellin may evade the recognition by TLR5 and has weak capability of inducing IL-8 which can recruit neutrophils, thereby its inflammatory activity is low [Andersen-Nissen et al., 2005]. Furthermore, after persistence colonization some *H. pylori* population is incline to delete their cag genes [Akopyants et al., 1998], which may due to $cagA^+$ inducing an enhanced IL-8 level in human tissue [Peek et al., 1995; Kim et al., 2006]. In our study, *H. pylori* 26695 *ΔspoT* strain expressed higher level of *cagA* protein (Fig. 4B), and human gastric epithelial AGS infected by this mutant strain excreted more IL-8 (Fig. 4A) comparing to that infected by the wild-type. In addition, *napA* is violent expression (Fig. 4B), as is chemotactic for human neutrophils and monocytes [Brisslert et al., 2005], which hints that *H. pylori* 26695 (*ΔspoT*) may induce more neutrophils. Activated neutrophils will produce ROS/RNS to kill the bacteria [Handa et al., 2010], so *H. pylori* 26695 (*ΔspoT*) may facing more reactive oxygen stress. Reactive oxygen stress may be an important reason for the poor survival ability of the *H. pylori* 26695 *ΔspoT* strain while exposed to SNP (Fig. 5) and can explain why *ΔspoT* strain elicits decreased colonization in the mice stomach.

In summary, in this study we investigated the anti-oxidative stress function of the *H. pylori SpoT* protein in vitro and its roles in colonization in vivo. Our results showed that the *H. pylori spoT* gene may affect its energy metabolism, detoxification and protein fate and so on to adapt to the atmosphere oxygen tension. Moreover, *SpoT* is critical for *H. pylori* colonization in mice without inducing strong immune response.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Nos. 81071313, 30800037, 81171536, and 30972775, 30971151), the Science Foundation of Shandong Province, China (Nos. ZR2009CZ001, ZR2009CM002, BS2010YY038, BS2010YY040 and BS2011SW023) and the National Basic Research Program of China (973 Program 2012CB911202).

REFERENCES

Akopyants NS, Clifton SW, Kersulyte D, Crabtree JE, Youree BE, Reece CA, Bukanov NO, Drazek ES, Roe BA, Berg DE. 1998. Analyses of the cag pathogenicity island of *Helicobacter pylori*. Mol Microbiol 28:37–53.

Alamuri P, Mehta N, Burk A, Maier RJ. 2006. Regulation of the *Helicobacter pylori* Fe–S cluster synthesis protein NifS by iron, oxidative stress conditions, and fur. J Bacteriol 188:5325–5330.

Allen LA. 2000. Modulating phagocyte activation: The pros and cons of *Helicobacter pylori* virulence factors. J Exp Med 191:1451–1454.

Alm RA, Ling LS, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, deJonge BL. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature 397:176–180.

Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, Logan SM, Aderem A. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci USA 102:9247–9252.

Ando T, Kusugami K, Ohsuga M, Shinoda M, Sakakibara M, Saito H, Fukatsu A, Ichiyama S, Ohta M. 1996. Interleukin-8 activity correlates with histological severity in *Helicobacter pylori*-associated antral gastritis. Am J Gastroenterol 91(6):1150–1156.

Baker LM, Raudonikiene A, Hoffman PS, Poole LB. 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: Genetic and kinetic characterization. J Bacteriol 183(6):1961–1973.

Barnard FM, Loughlin MF, Fainberg HP, Messenger MP, Ussery DW, Williams P, Jenks PJ. 2004. Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen *Helicobacter pylori*. Mol Microbiol 51:15–32.

Blaser MJ, Atherton JC. 2004. *Helicobacter pylori* persistence: Biology and disease. J Clin Invest 113:321–333.

Bliss CM, Jr., Golenbock DT, Keates S, Linevsky JK, Kelly CP. 1998. *Helicobacter pylori* lipopolysaccharide binds to CD14 and stimulates release of interleukin-8, epithelial neutrophil-activating peptide 78, and monocyte chemotactic protein 1 by human monocytes. Infect Immun 66:5357–5363.

Braeken K, Moris M, Daniels R, Vanderleyden J, Michiels J. 2006. New horizons for (p)ppGpp in bacterial and plant physiology. Trends Microbiol 14:45–54.

Brisslert M, Enarsson K, Lundin S, Karlsson A, Kusters JG, Svennerholm AM, Backert S, Quiding-Jarbrink M. 2005. *Helicobacter pylori* induce neutrophil transendothelial migration: Role of the bacterial HP-NAP. FEMS Microbiol Lett 249:95–103.

Butler SM, Festa RA, Pearce MJ, Darwin KH. 2006. Self-compartmentalized bacterial proteases and pathogenesis. Mol Microbiol 60:553–562.

Casanova JL, Abel L, Quintana-Murci L. 2011. Human TLRs and IL-1Rs in host defense: Natural insights from evolutionary, epidemiological, and clinical genetics. Annu Rev Immunol 29:447–491; Review.

Cashel M, Gentry DR, Hernandez VJ, Vinella D. 1996. The stringent response. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella*: Cellular and molecular biology, 2nd edition. Washington, DC: ASM Press. pp. 1458–1496.

Chuang MH, Wu MS, Lin JT, Chiou SH. 2005. Proteomic analysis of proteins expressed by *Helicobacter pylori* under oxidative stress. Proteomics 5:3895–3901.

Comtois SL, Gidley MD, Kelly DJ. 2003. Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*. Microbiology 149:121–129.

Durfee T, Hansen AM, Zhi H, Blattner FR, Jin DJ. 2008. Transcription profiling of the stringent response in *Escherichia coli*. J Bacteriol 190:1084–1096.

Ernst FD, Homuth G, Stoof J, Mäder U, Waidner B, Kuipers EJ, Kist M, Kusters JG, Bereswill S, van Vliet AH. 2005. Iron-responsive regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur. J Bacteriol 187:3687–3692.

Ferrero RL. 2005. Innate immune recognition of the extracellular mucosal pathogen, *Helicobacter pylori*. Mol Immunol 42:879–885.

Gaynor EC, Wells DH, MacKichan JK, Falkow S. 2005. The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. Mol Microbiol 56:8–27.

Gottesman S. 1996. Proteases and their targets in *Escherichia coli*. Annu Rev Genet 30:465–506.

Handa O, Naito Y, Yoshikawa T. 2010. *Helicobacter pylori*: A ROS-inducing bacterial species in the stomach. Inflamm Res 59:997–1003.

Harris AG, Hinds FE, Beckhouse AG, Kolesnikow T, Hazell SL. 2002. Resistance to hydrogen peroxide in *Helicobacter pylori*: Role of catalase (KatA) and Fur, and functional analysis of a novel gene product designated 'KatA-associated protein', KapA (HP0874). Microbiology 148:3813–3825.

Harris AG, Wilson JE, Danon SJ, Dixon MF, Donegan K, Hazell SL. 2003. Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the *Helicobacter pylori* SS1 mouse model. Microbiology 149:665–672.

He Q, Wang JP, Osato M, Lachman LB. 2002. Real-time quantitative PCR for detection of *Helicobacter pylori*. J Clin Microbiol 40:3720–3728.

Hughes NJ, Chalk PA, Clayton CL, Kelly DJ. 1995. Identification of carboxylation enzymes and characterization of a novel four subunit pyruvate:-flavodoxin oxidoreductase from *Helicobacter pylori*. J Bacteriol 177:3953–3959.

Hughes NJ, Clayton CL, Chalk PA, Kelly DJ. 1998. *Helicobacter pylori* porCDAB and oorDABC genes encode distinct pyruvate:flavodoxin and 2-oxoglutarate:acceptor oxidoreductases which mediate electron transport to NADP. J Bacteriol 180:1119–1128.

Kelly DJ. 1998. The physiology and metabolism of the human gastric pathogen *Helicobacter pylori*. Adv Microb Physiol 40:137–189.

Kim SY, Lee YC, Kim HK, Blaser MJ. 2006. *Helicobacter pylori* CagA transfection of gastric epithelial cells induces interleukin-8. Cell Microbiol 8:97–106.

Kumar Pachathundikandi S, Brandt S, Madassery J, Backert S. 2011. Induction of TLR-2 and TLR-5 expression by *Helicobacter pylori* switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-α. PLoS ONE 6:e19614.

Kvint K, Hosbond C, Farewell A, Nybroe O, Nystrom T. 2000. Emergency derepression: Stringency allows RNA polymerase to override negative control by an active repressor. Mol Microbiol 35:435–443.

Loughlin MF, Arandhara V, Okolie C, Aldsworth TG, Jenks PJ. 2009. *Helicobacter pylori* mutants defective in the clpP ATP-dependant protease and the chaperone clpA display reduced macrophage and murine survival. Microb Pathog 46:53–57.

Mittenhuber G. 2001. Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). J Mol Microbiol Biotechnol 3:585–600.

Mouery K, Rader BA, Gaynor EC, Guillemin K. 2006. The stringent response is required for *Helicobacter pylori* survival of stationary phase, exposure to acid, and aerobic shock. J Bacteriol 188:5494–5500.

Muotiala A, Helander IM, Pyhälä L, Kosunen TU, Moran AP. 1992. Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infect Immun 60:1714–1716.

Nicholls P, Fita I, Loewen PC. 2001. Enzymology and structure of catalases. Adv Inorg Chem 51:51–106.

Ohkusa T, Okayasu I, Miwa H, Ohtaka K, Endo S, Sato N. 2003. *Helicobacter pylori* infection induces duodenitis and superficial duodenal ulcer in *Mongolian gerbils*. Gut 52(6):797–803.

Olczak AA, Olson JW, Maier RJ. 2002. Oxidative stress resistance mutants of *Helicobacter pylori*. J Bacteriol 184:3186–3193.

Olczak AA, Wang G, Maier RJ. 2005. Up-expression of NapA and other oxidative stress proteins is a compensatory response to loss of major *H. pylori* stress resistance factors. Free Radic Res 39:1173–1182.

Patel MS, Roche TE. 1990. Molecular biology and biochemistry of pyruvate dehydrogenase complexes. FASEB J 4:3224–3233.

Peek RM, Jr., Miller GG, Tham KT, Perez-Perez GI, Zhao X, Atherton JC, Blaser MJ. 1995. Heightened inflammatory response and cytokine expression in vivo to cagA$^+$ *Helicobacter pylori* strains. Lab Invest 73:760–770.

Qu W, Zhou Y, Shao C, Sun Y, Zhang Q, Chen C, Jia J. 2009. *Helicobacter pylori* proteins response to nitric oxide stress. J Microbiol 47:486–493.

Robertson GT, Ng WL, Gilmour R, Winkler ME. 2003. Essentiality of clpX, but not clpP, clpL, clpC, or clpE, in *Streptococcus pneumoniae* R6. J Bacteriol 185:2961–2966.

Sharma SA, Tummuru MK, Miller GG, Blaser MJ. 1995. Interleukin-8 response of gastric epithelial cell lines to *Helicobacter pylori* stimulation in vitro. Infect Immun 63(5):1681–1687.

Wells DH, Gaynor EC. 2006. *Helicobacter pylori* initiates the stringent response upon nutrient and pH downshift. J Bacteriol 188:3726–3729.

Wickner S, Maurizi MS, Gottesman S. 1999. Posttranslational quality control: Folding, refolding, and degrading proteins. Science 286:1888–1893.

Zhou YN, Coleman WG, Jr., Yang Z, Yang Y, Hodgson N, Chen F, Jin D. 2008. Regulation of cell growth during serum starvation and bacterial survival in macrophages by the bifunctional enzyme *SpoT* in *Helicobacter pylori*. J Bacteriol 190:8025–8032.

Zu Y, Cassai ND, Sidhu GS. 2000. Light microscopic and ultrastructural evidence of in vivo phagocytosis of *Helicobacter pylori* by neutrophils. Ultrastruct Pathol 24:319–323.