RESEARCH ARTICLE

Phosphoproteome analysis of the pathogenic bacterium *Helicobacter pylori* reveals over-representation of tyrosine phosphorylation and multiply phosphorylated proteins

Ruiguang Ge[1], Xuesong Sun[2]*, Chuanle Xiao[2], Xingfeng Yin[2], Weiran Shan[1], Zhuo Chen[1] and Qing-Yu He[2]***

[1] The Laboratory of Integrative Biosciences, College of Life Sciences, Sun Yat-Sen University, Guangzhou, P. R. China
[2] Institute of Life and Health Engineering and National Engineering Research Center for Genetic Medicine, Jinan University, Guangzhou, P. R. China

Received: October 12, 2010
Revised: January 4, 2011
Accepted: January 18, 2011

Increasing evidence shows that protein phosphorylation on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues is a major regulatory post-translational modification in the bacteria. To reveal the phosphorylation state in the Gram-negative pathogenic bacterium *Helicobacter pylori*, we carried out a global and site-specific phosphoproteomic analysis based on TiO_2-phosphopeptide enrichment and high-accuracy LC-MS/MS determination. Eighty-two phosphopeptides from 67 proteins were identified with 126 phosphorylation sites, among which 79 class I sites were determined to have a distribution of 42.8:38.7:18.5% for the Ser/Thr/Tyr phosphorylation, respectively. The *H. pylori* phosphoproteome is characterized by comparably big size, high ratio of Tyr phosphorylation, high abundance of multiple phosphorylation sites in individual phosphopeptides and over-representation of membrane proteins. An interaction network covering 28 phosphoproteins was constructed with a total of 163 proteins centering on the major *H. pylori* virulence factor VacA, indicating that protein phosphorylation in *H. pylori* may be delicately controlled to regulate many aspects of the metabolic pathways and bacterial virulence.

Keywords:
Helicobacter pylori / Membrane / Microbiology / Phosphoproteome / Tyrosine phosphorylation

1 Introduction

Protein phosphorylation is the most abundant and biologically the most important post-translational modification. It is perhaps the best studied due to the close association of dys-regulated phosphorylation with human pathologies [1]. Catalyzed in a reversible fashion by specific protein kinases and phosphatases, phosphorylation processes produce fast and precise changes in protein properties, in turn affecting many critical processes, including protein–protein interactions, cell signaling, cytoskeleton remodeling, cell-cycle events and cell–cell interactions [2]. Therefore, it is extremely important to determine the degree and the site of the

Correspondence: Dr. Ruiguang Ge, College of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, P. R. China
E-mail: gerg@mail.sysu.edu.cn
Fax: +86-20-39332973

Abbreviations: CagA, cytotoxin-associated gene A; **Flg,** flagella; **FPR,** false positive rate; **GO,** gene ontology; **LTQ,** linear ion trap; **PIM,** protein interaction map; **Ser,** serine; **Thr,** threonine; **Tyr,** tyrosine

*These authors have contributed equally to this study.
**Additional corresponding author: Professor Qing-Yu He
E-mail: tqyhe@jnu.edu.cn

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

in vivo protein phosphorylation. However, due to the very low stoichiometry, limited dynamic range, high complexity and quantitative difficulties of protein phosphorylations, highly selective enrichment procedures and sensitive MS are required to decipher the phosphoproteome [3]. Selective phosphopeptide enrichment has been accomplished in several ways by using antiphosphotyrosine antibodies, immobilized metal affinity chromatography (IMAC), chemical modifications or strong cation exchange chromatography [4]. The seamless combination of IMAC and nano-LC enables reproducible separation and identification of phosphopeptides in a low-femtomole range [5, 6], and thus it is the most frequently used method in the study of cellular phosphorylation.

As a Gram-negative, spiral-shaped bacterium that colonizes in the gastric mucosa of humans [7], *Helicobacter pylori* has been frequently associated with atrophic gastritis, peptic ulcer disease, functional dyspepsia and gastric carcinomas [8]. *H. pylori* strains can be classified according to the presence or the absence of the two virulence factors, i.e. an active vacuolating toxin VacA [9], and a 40-kbp *cag* pathogenicity island (*cag* PAI) encoding a 120–145 kDa cytotoxin-associated gene A (CagA) as well as the respective type IV secretion system [10]. VacA is proposed to play a role in the early steps of bacterial gastric colonization by provoking cell vacuolation and inducing gastric cell apoptosis [11]. CagA protein is an important factor in the clinical outcome of gastritis treatment, and therefore has been extensively studied during the past two decades [12]. Translocated CagA protein undergoes tyrosine (Tyr) phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif in the host cells and in turn induces a cellular hummingbird phenotype of transformation [13]. Nonphosphorylated CagA interacts with host proteins, such as epithelial tight junction-scaffolding protein zonulin (ZO-1), cell adhesion protein E-cadherin, hepatocytes growth factor receptor c-Met, cadherin-associated protein β-catenin, adaptor protein GRB-2 and the kinase PAR1, leading to a loss of cell polarity and inducing pro-inflammatory and mitogenic responses [14].

Besides the knowledge about the phosphorylation of secreted CagA in the gastric epithelia cells, there is not much information available about the in vivo phosphorylation state of *H. pylori* proteins. Inspection of the genome of *H. pylori* 26695 revealed that the bacterium contains at least one protein kinase (HP0432) and one PPM-family protein phosphatase (HP0431) [15]. One attempt to globally analyze the phosphoproteome of *H. pylori* 26695 with SDS-PAGE and autoradiography revealed eight proteins phosphorylated at serine (Ser) residues [16]. Another study identified 57 proteins through Fe^{3+}-IMAC enrichment, 2-D gel electrophoresis and MALDI-TOF MS analysis, without further phosphorylation site mapping [17]. Here, we globally analyzed the Ser/threonine (Thr)/Tyr phosphoproteome of *H. pylori* 26695 through the combined use of IMAC phosphopeptide enrichment and high-accuracy MS. In total, 82 phosphopeptides from 67 proteins were identified with 126 phosphorylation sites (class I/II/III). For the identified phosphopeptides, 79 phosphorylation sites were determined to be class I (with localization probability higher than 0.75): 33 (42.8%) on Ser, 31 (38.7%) on Thr and 15 (18.5%) on Tyr. The *H. pylori* phosphoproteome was then characterized and discussed based on the properties of the identified phosphoproteins, which will provide valuable clues for further investigations regarding the bacterial phosphorylation and the pathology of *H. pylori*.

2 Materials and methods

2.1 Bacterial culture

The model cell of *H. pylori* 26695 was cultured as reported previously [18]. Briefly, *H. pylori* 26695 was maintained on Brucellar agar (Difco) plates supplemented with 10% v/v sheep blood at 37°C. The plates were incubated in an anaerobic jar fitted with a microaerobic gas-generating kit (Oxoid). Cells resuspended in 0.9% w/v NaCl were diluted into the basal medium of Brucellar broth (Difco) containing 5% v/v FBS (GIBCO) for large-scale bacterial culture.

2.2 Preparation and digestion of cellular proteins

The harvested cells were washed thrice with prechilled PBS (10 mM, pH 7.4), and then were disrupted in a lysis buffer containing 15 mM Tris-HCl (pH 8.0), 7 M urea, 2 M thiourea, 1% DTT, 4% CHAPS and 5 mM each of the following protein phosphatase inhibitors: sodium fluoride, 2-glycerol phosphate, sodium vanadate and sodium pyrophosphate. The mixture was frozen–thawed thrice and then sonicated six times each for 30 s. The lysate was centrifuged at $12\,000 \times g$ for 10 min at 4°C and the supernatant was recovered. Protein concentrations were determined using the Bradford assay.

An equivalent of 10 mg total protein extracts was reduced with 10 mM DTT at 37°C, and then alkylated with 20 mM iodoacetamide at room temperature in the dark for 45 min. Proteins were buffer exchanged into 25 mM ammonium bicarbonate by a PD-10 desalting column (GE Healthcare) and then digested with sequence-grade trypsin (1:100 w/w) (Promega) overnight at 37°C. These digested peptides were dried in a SpeedVac (Thermo Electronic) to remove any partial salts of ammonium bicarbonate.

2.3 Enrichment of phosphopeptides by immobilized metal affinity chromatography

Phosphorylated tryptic peptides in the resulting in-solution digests were enriched using the ProteoExtract Phosphopeptide

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

TiO_2 Enrichment kit (Calbiochem) according to the protocol provided by the manufacturer. Briefly, 50 μL of TiO_2 resin was mixed with the tryptic digests previously redissolved in 200 μL of TiO_2 Phosphobind buffer (as provided in the kit) containing 50 g/L DHB. After 30-min incubation, the supernatant was discarded, and the resin was washed thrice with the Wash Buffer (as provided by the kit). Then, the phosphopeptides were eluted twice by the Elution Buffer. The eluates were combined and dried using the SpeedVac (Thermo Fisher).

2.4 Phosphorylation site mapping by LC-MS/MS

Enriched phosphopeptides were dissolved in 5% ACN/0.1% formic acid and analyzed with a Finnigan Surveyor HPLC system coupled online with a linear ion trap (LTQ)-Orbitrap mass spectrometer (Thermo Electron) equipped with a nanospray source [19]. Briefly, the peptides were eluted from a C18 column (100 μm id, 10 cm long, 5 μm resin from Michrom Bioresources) using the following gradient conditions: 5∼36.5% ACN, 0.1% formic acid over 90 min, and monitored online with the LTQ-Orbitrap [20]. The general parameters used for the MS analysis were as follows: the spray voltage of 1.80 kV; zero sheath and auxiliary gas flow; the ion transfer tube temperature of 200°C; an ion selection threshold of 1000 counts for MS^2 and 500 counts for MS^3; activation Q of 0.25, activation time of 30 ms, isolation window of $m/z = 3$, normalized collision energy of 35% and one micro-scan for MS^2; resolution R of 60 000 for MS; and dynamic exclusion time of 90 s. The mass spectrometer was set to operate in the positive ion mode and employed a data-dependent automatic switch between MS and MS^2 acquisition modes. For each cycle, one full MS scan in the Orbitrap at AGC target of 1×10^6 and maximum ion accumulation time of 500 ms was followed by the top five MS^2 scans in the LTQ at AGC target of 5000 and maximum ion accumulation time of 100 ms. For phosphopeptide detection, MS^3 was triggered if a neutral loss peak at either −98.0, −49.0, −32.7 or −24.5 was identified in the MS^2 and the peak was one of the five most intense ions [21].

2.5 Data processing

Database searching for the collected MS and MS^2 data was carried out using the MASCOT search engine (Matrix Science, London, UK) against the concatenated forward and reverse *H. pylori* 26695 genome sequence database. MASCOT was configured to allow for the post-translational modifications of carbamidomethylation (fixed), oxidation of methionine and phosphorylation of Ser, Thr and Tyr residues. Initial mass deviation of precursor ions and fragment ions was allowed up to 10 ppm and 0.5 Da, respectively. The false-positive rate (FPR) of peptide identification was estimated according to the following formula: $\% \text{ FPR} = 2[n_{rev}/(n_{rev}+n_{real})]$ [22], where % FPR is the estimated false-positive rate, n_{rev} is the number of peptides hits matched to the reverse database and n_{real} is the number of the peptides identified from the real database. The fragmentation spectra of potential phosphopeptides were manually verified for both the presence of a phosphate group and the peptide sequence using the criteria as described by Mann and coworkers [23]. The probabilities for the potential site of phosphorylation were calculated from the post-translational modification scores in the MSQuant [24], and only phosphorylation sites categorized as class I (with probability over 0.75) or class II/III sites (with probability between 0.25 and 0.75) were reported.

2.6 Bioinformatics analysis

As there is no gene ontology (GO) annotation available for the *H. pylori* proteome, the information on cellular function and localization of the identified phosphoproteins was obtained by searching against the Swiss-Prot database by the Blast2GO tool [25] or the bacterial localization prediction tool pSORTb (version 3.0.0) [26]. In total, 782 *H. pylori* proteins were annotated and classified as the reference data set; the test data set for the enrichment analysis was the list of the identified phosphoproteins. The algorithm of Fisher's Exact Test was used to find statistically over-represented GO categories among the identified phosphoproteins. All GO terms were selected as over-represented with $p < 0.05$. With the availability of the complete DNA sequence [27], as well as the large-scale protein–protein interaction map (PIM) of *H. pylori* [28], the candidate proteins with the potential to interact with the identified phosphoproteins were searched against PIMRider (http://pim.hybrigenics.com), a web-based protein–protein interaction predication software based on two-hybrid assays. According to the method of Rain et al. [28], a PIM was calculated for every interaction with biological score ranging from 0 (specific interaction) to 1 (probable artifact). For practical use, the scores were divided into four categories: from A (score very close to 0) to D (close to 1).

3 Results and discussion

3.1 Phosphoproteome of *H. pylori*

For a global view of the *H. pylori* Ser/Thr/Tyr phosphoproteome, proteins from the cells were extracted, denatured and digested with trypsin in solution. Phosphopeptides were separated and enriched from the resulting complex peptide mixtures using IMAC, and then analyzed thrice on a high-accuracy LTQ-Orbitrap mass spectrometer to improve phosphopeptide coverage and confidence of identification. Figure 1 shows a representative MS/MS spectrum acquired in the LTQ mass spectrometer of a phospho-Ser peptide IFPSPSLpSMTTIVNEHAKELR from the phosphoserine aminotransferase (SerC). Eighty-four phosphopeptides were

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

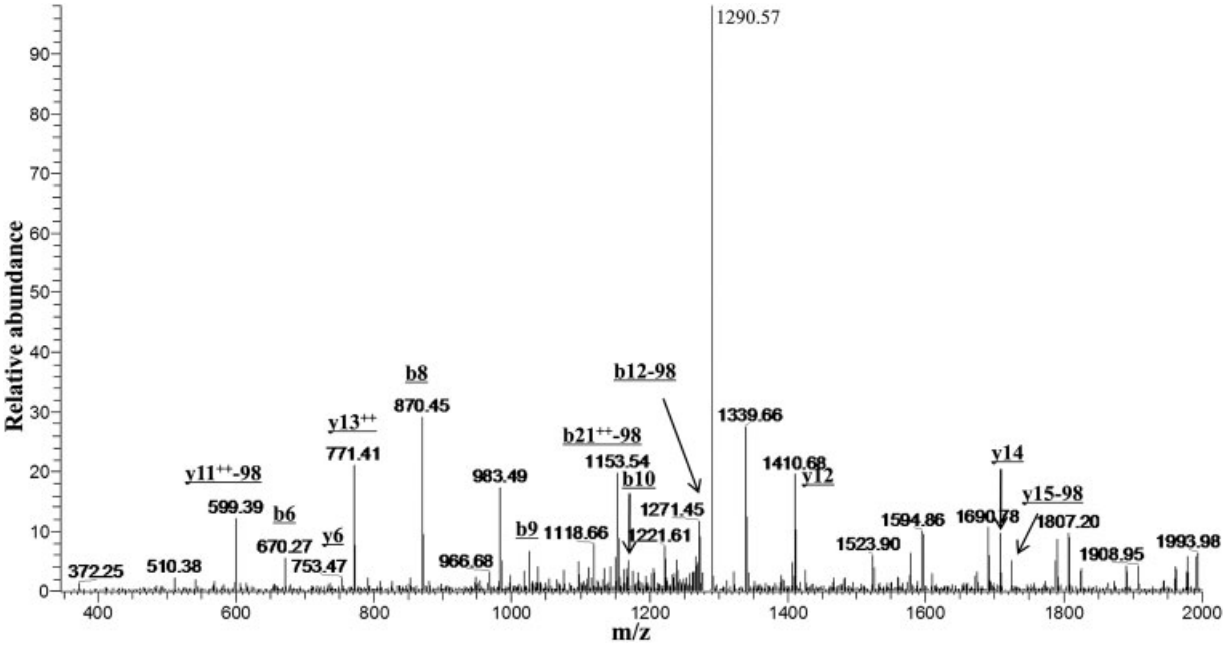

Figure 1. A MS/MS spectrum acquired in the LTQ mass spectrometer of a phospho-Ser peptide lFPSPSLpSMTTIVNEHAKELR from the phosphoserine aminotransferase (SerC).

identified from 67 proteins with an estimated FPR of $<1\%$. In total, 126 phosphorylated sites were identified, and among them 79 were determined to have a localization probability for the phosphate higher than 0.75 (class I phosphorylation site). The distribution between individually identified class I sites was determined to be 33 (42.8%) phospho-Ser, 31 (38.7%) phospho-Thr and 15 (18.5%) phospho-Tyr sites.

Table 1 compares the known phosphoproteomes from some bacterial species. Similar to the phosphoproteome of *Streptococcus pneumoniae* [19], *H. pylori* phosphoproteome is characteristic by the high level of multiple phosphorylation sites in one single peptide. Another characteristic derived from the direct comparison is the high ratio of Tyr phosphorylation in *H. pylori* phosphoproteome. All the identified phosphopeptides with their protein accession numbers, gene annotations and identified phosphosites are listed in Table 2. The spectra for each of the phosphopeptides are listed in the Supporting Information material as per the standards for the identification of phosphorylation sites [29], which can be accessible from the following website: http://life-health.jnu.edu.cn/phospho/MS_data_for_peer_review.pdf.

3.2 Classification of phosphorylated proteins

Phosphorylated proteins identified in this study are involved in a wide variety of bacterial metabolic and regulatory processes. Consistent with the GO enrichment analysis, phosphoproteins located in the membrane (either cytoplasmic or outer membrane) of *H. pylori* seemed to be over-represented, whereas cytoplasmic proteins were under-represented with respect to the whole proteome. Among the 67 identified *H. pylori* proteins, 13 were proposed to be located in the cytoplasm by the bacterial protein subcellular localization prediction program pSORTb (version 3.0.0), eleven on the cytoplasmic membrane, seven on the outer membrane, one on the periplasm and one as extracellular-localized protein (Fig. 2). However, we failed to obtain the localization information for around 50% of the identified phosphoproteins.

Among the 67 identified phosphoproteins, 23 (34%) were annotated as hypothetical proteins in the Swiss-Prot database, making it difficult to deduce their respective biological functions. Therefore, we submitted all the identified phosphoproteins to the automated BLASTP, InterPro and GO analysis. Forty-four phosphoproteins were successfully annotated with regard to their respective involvement in the biological processes or molecular functions. There exists a significant amount of redundancy among the biological processes for the phosphoproteins (95 biological processes *versus* 44 phosphoproteins): 29 in metabolic processes, 40 in (regulation of) cellular processes, 16 in localization, 3 in locomotion, 4 in response to stimulus, 2 in development processes and 1 in multiorganism process. Identified phosphoproteins were significantly over-represented in the main pathways of nucleoside binding ($p = 1.8$ E-3 by Fisher's exact test), pyridoxine biosynthetic/metabolic process ($p = 5.4$ E-3) and vitamin B6 biosynthetic process ($p = 5.4$ E-3).

As for the category of molecular functions, the majority of proposed functions included enzymatic or catalytic activity (35 in total; including transferase (13), hydrolase (12), oxidoreductase (4) and isomerase (3)) as well as

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Phosphoproteome comparison between *H. pylori* and several other bacteria

Bacterium[a)]	No. of phosphopeptides	No. of phosphosites	pS (%)	pT (%)	pY (%)
E. coli [23]	105	81	67.9	23.5	8.6
B. subtilis [50]	103	78	69.2	20.5	10.3
L. lactis [44]	102	79	46.5	50.6	2.7
P. putida [32]	56	53	52.8	39.6	7.5
P. aeruginosa [32]	57	55	52.7	32.7	14.5
Campylobacter jejuni [17]	58	35	30.3	72.7	9.1
S. pneumoniae [19]	102	163	47.2	43.8	9.0
S. coelicolor [30]	44	44	34.1	52.3	13.6
K. pneumoniae [31]	117	93	31.2	15.1	25.8
H. pylori[b)]	80	124	42.8	38.7	18.5

a) Bacterial name followed by reference in brackets.
b) The Ser/Thr/Tyr phosphorylation ratio is determined from the 79 class I sites.

molecular interactions (28 in total; with proteins (5), nucleic acid (8), cofactors (6), nucleotide (8), nucleoside (2), ions (6), vitamin (3) and lipids (3)). There are also proteins related to transporter (4), structural molecule (2), molecular transducer (1) and electron carrier (1) activities. BLASTP and InterPro analyses resulted in the annotation of only two sequences previously described as hypothetical proteins (HP0468 and HP0112), indicating that quite limited entries have been added into the Swiss-Prot database ever since the completion of the genome project over 10 years ago [27]. Therefore, the efforts are urgently needed to discover the biological functions of these kinds of hypothetical proteins.

3.3 High level of Tyr phosphorylation and membrane protein phosphorylation

A noteworthy feature of *H. pylori* phosphoproteome is the significantly high overall abundance (~18.5%) of Tyr phosphorylation, whereas most other bacterial model organisms with known phosphoproteomes have lower than 10% phospho-Tyr sites (Table 1). There are three bacterial phosphoproteomes with comparable Tyr phosphorylation percentage. The exponentially growing *Streptomyces coelicolor* has relative levels of Ser, Thr and Tyr phosphorylations with ~34, 52 and 14%, respectively [30]. Among the identified 93 distinct phosphorylation sites in *Klebsiella pneumoniae*, there are 24 Tyr phosphorylation sites (~25.8%) [31]. *Pseudomonas aeruginosa* phosphoproteome is another one with comparable Tyr phosphorylation level (14.5%), whereas the nonpathogenic *P. putida* species has a much lower Tyr phosphorylation level (7.5%) [32]. The level of Tyr phosphorylation seems to be positively correlated with the pathogenicity. In fact, various findings have supported the contribution of Tyr phosphorylation to the bacterial pathogenicity: pedestal formation (Tir of enteropathogenic *Escherichia coli* and *Citrobacter*) [33]; cell elongation, scattering and inflammation (CagA of *Helicobacter*) [13, 34]; capsular polysaccharide biosynthesis [31]; cell invasion (Tarp of *Chlamydia*) [35]; and proinflammatory responses and cell proliferation (BepD-F of *Bartonella*) [36]. In this study, we focused on the determination of the intracellular phosphoproteome, and therefore CagA was not found to be present in this phosphoproteome as CagA is Tyr-phosphorylated when injected into the gastric epithelial cells.

Another feature of *H. pylori* phosphoproteome is the over-representation of membrane proteins. Several are supposed to be the cell envelope proteins or to be involved in the production of cell envelope component, including outer membrane protein P1 (OmpP1), phospho-*N*-acetylmuramoyl-pentapeptide transferase (MraY), alginate *O*-acetylation protein (Algl), lipooligosaccharide 5G8 epitope biosynthesis-associated protein (Lex2B), LPS biosynthesis protein, spore coat polysaccharide biosynthesis protein C (WecE), flagella hook (FlgE) and flagellin B homolog (FlgB). Membrane proteins, involved in the pathways of peptidoglycan-based cell-wall biogenesis, peptidoglycan biosynthesis/metabolism, cell wall biogenesis, transitional metal ion transmembrane transporter, di-/tri-valent inorganic cation transmembrane transporter, were determined to be over-represented through GO enrichment analysis.

Striking evidence has been established for the association between protein Tyr phosphorylation and the control of surface polysaccharide production or transport, as well as between the protein Ser/Thr phosphorylation and the bacterial virulence associated with cell-wall component [37]. Surface polysaccharides are believed to be involved in the early steps of the infection process and are considered potent virulent factors [38]. Some proteins from various species showed similar autophosphorylation activities while at the same time are also homologous to a family of enzymes involved in exopolysaccharide synthesis grouped together as BY-kinases (Bacterial tYrosine kinases) [39, 40]. For instance, the Tyr kinase Wzc of *E. coli* is essential for the synthesis of the exopolysaccharide colonic acid and the assembly of group 1 capsular polysaccharide [37]. There is no information available yet about the presence of Tyr kinases in *H. pylori* [37], except the identified involvement of CagA in the Tyr dephosphorylation

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. List of identified phosphopeptides from *H. pylori* 26695 categorized into putative TGIR functional groups [27]

Acc. (GI) number	Annotation		Peptide sequence[a)]	Phosphorylated residue[b)]
Amino-acid biosynthesis				
2313818	*hyuA*	Hydantoin utilization protein A (HP0695)	MN(p)T(p)TILEAYAAEP(p)SR	1pST[S236, T225, T226]
2313507	*aroA*	3-Phosphoshikimate 1-carboxyvinyltransferase (HP0401)	VENpTAKNpSFKITPP(p)T(p)TIK	T56, S60, 1pT[T67, T68]
2314414	*aroE*	Shikimate 5-dehydrogenase (HP1249)	DMLIYQAALpSFEK	S214
2314079	*alr*	Alanine racemase, biosynthetic (HP0941)	pSAKANDApSEIpTALLNTIAYETIpSpTLpSK	S344, S351, S366, S369, T354, T367
2313177		Phosphoglycerate dehydrogenase (HP0096)	SIEAEIMVLNKVVIpTQEVLpSQLPKLK	S63, T58
2313858	*serC*	Phosphoserine aminotransferase (HP0736)	IFPKSPSLpSMTTIVNEHAKELR	S286
Biosynthesis of co-factors, prosthetic groups and carriers				
2314766	*pdxA*	Pyridoxal phosphate biosynthetic protein A (HP1583)	IpTPFYV(p)SM(p)SHDVGLAPLK	T243, 1pS[S248, S250]
Cell envelope				
2313971	*ompP1*	Outer membrane protein P1 (HP0839)	DpTNpTFRLGVTYMGKSLR	T497, T499
			D(p)TN(p)TFRLGV(p)TYMGK(p)SLR	2pST[S510, T497, T499, T505]
2313604	*mraY*	Phospho-*N*-acetylmuramoyl-pentapeptide transferase (HP0493)	INQQSNAGMSAKMKFGMLFILpSLIVpSVLLpSLK	S136, S140, S144
			INQQ(p)SNAGM(p)SAKMKFGMLFILpSLIV(p)SVLL(p)SLK	S136, 2pS[S119, S124, S140, S144]
			INQQ(p)SNAGM(p)SAKMKFGMLFIL(p)SLIV(p)SVLL(p)SLK	3pS[S136, S119, S124, S140, S144]
			INQQpSNAGM(p)SAKMKFGMLFIL(p)SLIV(p)SVLL(p)SLK	S119, 2pS[S136, S124, S140, S144]
2313988	*algI*	Alginate *O*-acetylation protein (HP0855)	NLEpSALKVLK	S424
2313960	*lex2B*	Lipooligosaccharide 5G8 epitope biosynthesis-associated protein (HP0826)	AY(p)SEGVGTQGYVITPKIAK	1pS[S185]
			AYSEGVG(p)TQGYVI(p)TPKIAK	1pT[T190, T196]
			AYSEGVGTQGYVIpTPKIAK	T196
2314763		LPS biosynthesis protein (HP1578)	ERWpSKPIKpTFFQK	S356, T361
2313467	*wecE*	Spore coat polysaccharide biosynthesis protein C (HP0366)	IFKDNPpYFpTPLHPLLKDK	T271, Y269
2323997	*flgE*	Flagellar hook (HP0870)	F(p)THATHAT(p)SIDVIDSLGpTKHAMR	T494, 1pST[S485, T478]
2313393	*flgB*	Flagellin B homolog (HP0295)	TMEAFKpTK	T92
Cellular processes				
2313163	*tlpC*	Methyl-accepting chemotaxis transducer (HP0082)	DLKTCVDNL(p)TK(p)TAHK	1pT[T504, T506]
2314023	*vacA*	Vacuolating cytotoxin (HP0887)	VNAAHNPL(p)S(p)THARVMMGGELK	1pST[S1244, T1245]
2313084	*groEL*	Chaperone and heat shock protein (HP0010)	VGGKEEI(p)TQVATI(p)SANSDHNIGK	1pST[S150, T144]
2314464	*secY*	Pre-protein translocase subunit (HP1300)	YLTILITLIQAVpSVpSVGLR	S119, S121
			YL(p)TILI(p)TLIQAV(p)SV(p)SVGLR	2pST[S119, S121, T109, T113]
			YLpTILIpTLIQAVSVSVGLR	T109, T113

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Continued

Acc. (GI) number	Annotation		Peptide sequence[a)]	Phosphorylated residue[b)]
2313655	*cag19*	*cag* PAI protein (HP0540)	QKpTAQAELKAIEAQ(p)S(p)SAK	T44, 1pS[S56, S57]
			QK(p)TAQAELKAIEAQ(p)S(p)SAK	2pST[S56, S57, T44]
2313091	*virB4*	virB4 homolog (HP0017)	NpYAPKLL(p)S(p)SK	Y180, 1pS[S186, S187]
Central intermediary metabolism				
2313524	*speA*	Arginine decarboxylase (HP0422)	GLN(p)YGLEAG(p)SK(p)SELIIAM(p)S(p)Y(p)TNPK	2pST[S115, S117, S124, T126], 1pY[Y109, Y125]
2313156	*ureC*	Urease protein (HP0075)	NAL(p)S(p)SQAVVATNM(p)SNLALK	1pS[S278, S279, S288]
Energy metabolism				
2314429	*nqo5*	NADH-ubiquinone oxidoreductase, NQO5 subunit (HP1262)	DLHKIAPpTILK	T260
2313455	*dxs*	Deoxyxylulose-5-phosphate synthase (HP0354)	KSKSAILSPTEApYSNTLLELAK	Y315
Fatty acid and phospholipid metabolism				
2313676	*fabF*	Beta ketoacyl-acyl carrier protein synthase II (HP0558)	RIVVpTGMGMINSLGLNK	T7
			RIVV(p)TGMGMIN(p)SLGLNK	1pST[S14, T7]
2313169	*fabD*	Malonyl coenzyme A-acyl carrier protein transacylase (HP0090)	DDLKALEPpTLKEMGAK	T179
Purines, pyrimidines, nucleosides and nucleotides				
2314710	*punB*	Purine nucleoside phosphorylase (HP1530)	KMVQKGVLLENMEFF(p)SVL(p)SVAK	1pS[S130, S133]
			KMVQKGVLLENMEFFpSVLSVAK	S130
Regulatory functions				
2313901	*spoT*	Penta-phosphate guanosine-3′-pyrophosphohydrolase (HP0775)	NECNIVGVpSpYLGYK	S725, Y726
Replication				
2313132		Adenine/cytosine DNA methyltransferase (HP0054)	pSAYFpTNPFIINEIAK	S445, T449
2314653	*uvrD*	DNA helicase II (HP1478)	(p)TL(p)T(p)SRLApYLIGVCGVP(p)SENTL(p)TL(p)TF(p)TNKA(p)SK	Y47, 4pST[S43, S56, S69, T40, T42, T61, T63, T65]
2314576	*hsdM*	Type I restriction enzyme M protein (HP1403)	QDKSIYDPACGSGpSLLLK	S195
2314695	*Eco57Ir*	Type IIS restriction enzyme R and M protein (HP1517)	LpYQELKQNPNK	Y703
Transcription				
2314357	*rpoB*	DNA-directed RNA polymerase, beta subunit (HP1198)	pYpTVREAMER	T81, Y80
Translation				
2314657	*pepQ*	Proline dipeptidase (HP1485)	pYSLEGKITEGFSDDGEPKG(p)S(p)SGMPMLSVLR	Y52, 1pS[S71, S72]
2314218	*prmA*	Ribosomal protein L11 methyltransferase (HP1068)	NFpSLNQIPLLVQDK	S238
2313378	*tgt*	tRNA-guanine transglycosylase (HP0281)	YpSKApYLHHLFRAK	S312, Y315

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Continued

Acc. (GI) number	Annotation		Peptide sequence[a)]	Phosphorylated residue[b)]
2313340	*deaD*	ATP-dependent RNA helicase, DEAD-box family (HP0247)	NNHTIEALVI(p)TP(p)TR	1pT[T94, T96]
Transport and binding proteins				
2314379	*yhcG*	ABC transporter, ATP-binding protein (HP1220)	FpSVPLKREFK	S116
2313342	*oppD*	Oligopeptide ABC transporter, ATP-binding protein (HP0250)	VAIVGEpSGpSGKSpSIANIIMR	S36, S38, S42
2314221	*copA*	Copper-transporting ATPase, P-type (HP1072)	pTGFGISAKTDYQGTK	T499
			(p)TGFGI(p)SAKTDYQG(p)TK	1pST[S504, T499, T512]
2313813	*feoB*	Iron(II) transport protein (HP0687)	KNLKNpSIVGR	S517
Other categories (adaptations and atypical conditions)				
2314413	*vacB*	Virulence associated protein homolog (HP1248)	(p)TDALILCQIEC(p)SKK	1pST[S92, T81]
Unknown function				
2313817		Conserved hypothetical integral membrane protein (HP0693)	LLKYLApSLPK	S91
2313572		Conserved hypothetical protein (HP0468)	GVSpYNENIALK	Y48
2314029		Conserved hypothetical protein (HP0891)	YEPKpTEVEIRR	T144
2314093		Conserved hypothetical protein (HP0956)	EINAPILpTFK	T146
2314751		Conserved hypothetical protein (HP1570)	IKLLLLDVDGpTLTDGSLYFDENFHEIK	T12
2313617		Conserved hypothetical secreted protein (HP0506)	pSKLHGK	S362
2313146		Predicated coding region HP0060	ID(p)TQRFG(p)SFMQN(p)SREAR	1pST[S498, S503, T493]
2313150		Predicated coding region HP0064	HVLNFNLEGLFIDFMQpSLK	S82
2313200		Predicated coding region HP0112	HNRLLPRDpYLGpYR	Y141, Y144
2313258		Predicated coding region HP0167	(p)TPPYLQN(p)TLDPQNANVPLEPK	1pT[T41, T48]
2313525		Predicated coding region HP0408	(p)TLpYAF(p)SLIDSQYCSK	Y108, 1pST[S111, T106]
2313558		Predicated coding region HP0448	LKEpSGVKDEELK	S49
2313600		Predicated coding region HP0488	WVIpTSYEIDK	T918
			WVITSpYEIDK	T920
2313625		Predicated coding region HP0513	YLH(p)TIGNL(p)TL(p)TGYN(p)SK	2pST[S444, T433, T438, T440]
2313711		Predicated coding region HP0586	DNApYVFRIKDLK	Y564
2313733		Predicated coding region HP0609	N(p)T(p)TFKGTNTLINSDPFSR	1pT[T1156, T1157]
2313893		Predicated coding region HP0766	LENMQDISLQSSHEVGVDITESKML(p)TK	1pT[T27]
2314015		Predicated coding region HP0880	LHKNKLpTIISK	T47
2314037		Predicated coding region HP0893	EILARAKDpTK	T76
2314110		Predicated coding region HP0963	EKEIALLpTLQEIQK	T81
2314184		Predicated coding region HP1028	FNLDEVLKpTIK	T159
2314210		Predicated coding region HP1055	(p)YFVS(p)YFGTRF(p)YGDLLLGGGALK	1pY[Y144, Y148, Y154]
			YFV(p)SYFG(p)TRFYGDLLLGGGALK	1pST[S147, T151]
2314291		Predicated coding region HP1127	KVDFPPYPGGSMIpSIQVNFTTKEEQ	S182

a) Class I phosphorylation sites are indicated by a lower case "p" in front of the amino acid, whereas class II and III phosphorylation sites are indicated by a parenthesized "p."
b) Ambiguous (class II and III) phosphorylation sites are presented in brackets with the total number of phosphorylation sites shown before.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

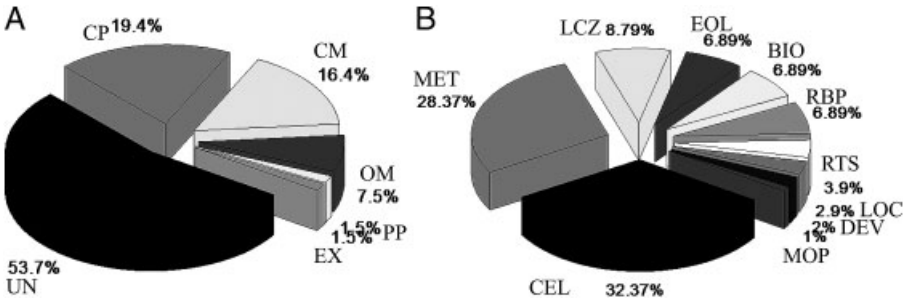

Figure 2. Classification of the identified phosphoproteins of *H. pylori* according to the cellular location (A) and biological process (B). The abbreviations used are as follows: CP, cytoplasmic; CM, cytoplasmic membrane; OM, outer membrane; PP, periplasmic; EX, extracellular; UN, unknown localization; CEL, cellular process; MET, metabolic process; LCZ, localization; EOL, establishment of localization; BIO, biological regulation; RBP, regulation of biological process; RTS, response to stimulus; LOC, locomotion; DEV, developmental process and MOP, multi-organism process.

of several host cell proteins [41]. Bioinformatics and experimental efforts in this regard are urgently needed to understand the biological regulations and functions of the presence of high ratio of Tyr phosphorylation in the pathogenesis of *H. pylori*.

Membrane proteins play important roles in various biological processes and are key players in bacterial virulence. YhcG, OppD, CopA and Feo, the four membrane proteins involved in metal ion transport, were here identified to be phosphorylated, indicating that phosphorylation takes part in the regulation of metal homeostasis. Among them, the *H. pylori* P-type ion pump CopA specifically binds and transports Cu^{2+} and Cd^{2+}, and the phosphorylation was predicted to lie between transmembrane segments H6 (amino acid positions 368–400) and H7 (amino acid positions 679–703) [42], which was unambiguously confirmed by the identification of one class I phosphorylation site at Thr-499 in this study.

3.4 High abundance of multiple phosphorylation sites

Another distinctive feature of *H. pylori* phosphoproteome is the high occurrence of multiple phosphorylation sites: 35 out of the identified 84 phosphopeptides containing at least two phosphorylation sites. One example is the peptide SAKANDASEITALLNTIAYETISTLSK from the enzyme of alanine racemase which was determined to be phosphorylated at six distinct residues. This phenomenon has been noted for the bacterial proteins under stress or overloading proteolytic conditions [43] and in the characterization of the phosphoproteome of the Gram-positive pathogenic bacterium *S. pneumoniae* [19] as well as the nonpathogenic *Lactococcus lactis* [44]. These observations indicated that one protein may be phosphorylated on multiple sites to fulfill the differential roles or to function together to achieve delicate micro-regulations of the virulence mechanisms, such as adhesion to the host, stimulation and regulation of pathogenic functions and impairing the host-defense mechanisms [45]. Multiple phosphorylation sites may have similar biological meaning, at least for the species of *L. lactis* [44] and *H. pylori*: (i) Similar to the case of *L. lactis*, the large number of proteins with multiple phosphorylation sites is in good agreement with our previously published gel-based proteomic studies of *H. pylori*, showing that at least eight out of the identified proteins are distributed over two to five spots all with equal molecular weights but different p*I*s [46, 47]. It is an established phenomenon for the contribution of post-translational modifications, especially phosphorylation to protein p*I* shifts [48]; and (ii) Both *L. lactis* and *H. pylori* have smaller genome sizes, simpler transcriptional machinery and fewer (two and three, respectively) sigma factors compared with other model bacteria such as *E. coli*, suggesting more regulation through post-translational modifications.

3.5 Protein kinase motifs in *H. pylori*

Since there is no information available for the substrate specificity of the kinase from *H. pylori* despite the fact that the bacterium contains at least one protein kinase and one PPM-family protein phosphatase through genome analysis [15], the potential kinases (mammalian, yeast and some kinds of model bacterial sources) responsible for the modification of the identified phosphopeptides were searched against the Phosida algorithm to annotate the phosphorylation motifs [49]. The most abundant motifs are those from CK1 (10.6%), NEK6 (7.1%), CK2 (6.5%), PKA and CAMK2 (2.9% each), similar to the findings by Mann et al. on the bacterium *Bacillus subtilis* [50], which indicated that there does not exist much similarity between structural determinants for the kinase action in bacteria and eukaryotes. NetPhosBac, a predictor developed under the phosphoproteomes of *E. coli* and *B. subtilis* for Ser/Thr phosphorylation sites in bacterial proteins [51], successfully predicted 29.4% of the Ser and Thr phosphorylation sites identified in this study. The low-prediction success rate is not surprising as *H. pylori* is a phylogenetically distinct member to *E. coli* and *B. subtilis*.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

3.6 Protein–protein interaction network of phosphorylated proteins

An interaction map with the involvement of phosphoproteins is important to understand the regulatory mechanisms of post-translational modifications in *H. pylori*. In this study, we constructed an interaction map of the identified phosphorylated proteins with a web-based *H. pylori* protein–protein interaction database, PIMRider [28]. As shown in Fig. 3, the phosphoprotein interaction map consisted of a large network covering 163 proteins among which there are 28 identified phosphoproteins. The major *H. pylori* virulence factor VacA that can induce cytoplasmic vacuolation in cultured epithelial cell is centered on this interaction map. The possible partners for VacA in the A category include an outer membrane porin protein HopL (PIM biological score, 1e−63), a hypothetical protein HP0699 (1e−22), a predicted ABC transporter HP1464 (1e−42) and a predicted ATPase/DNA transfer protein VirB4_5 (1e−500). The interaction between VacA and VirB4_5 has been verified previously as VacA is secreted from *H. pylori* through the syringe-like VirB/VirD4-like type IV secretion apparatus. It is worth noting that VirB4_5 is also identified to be phosphorylated, implicating the involvement of phosphorylation in the regulation of VacA secretion through the bacterial membrane. During the secretion from cytoplasm across the bacterial membrane and the plasma membrane of gastric epithelia cells, the VacA preprotein (~140 kDa) is cleaved into the mature toxin subunits of around 88 kDa [52]. The mature toxin subunits can aggregate into oligomeric complexes for the subsequent insertion into lipid bilayers to form anion-selective channels [53]. Although there is no information available to verify the interaction between VacA with HopL and HP1464, these proteins may be involved in the reported retainment of VacA on the surface of the bacterium as per their respective TGIR functional groups [27, 54].

GroEL and FlgB are the two partner proteins that are determined to be phosphorylated and predicted to interact directly with each other. The in vivo interaction between

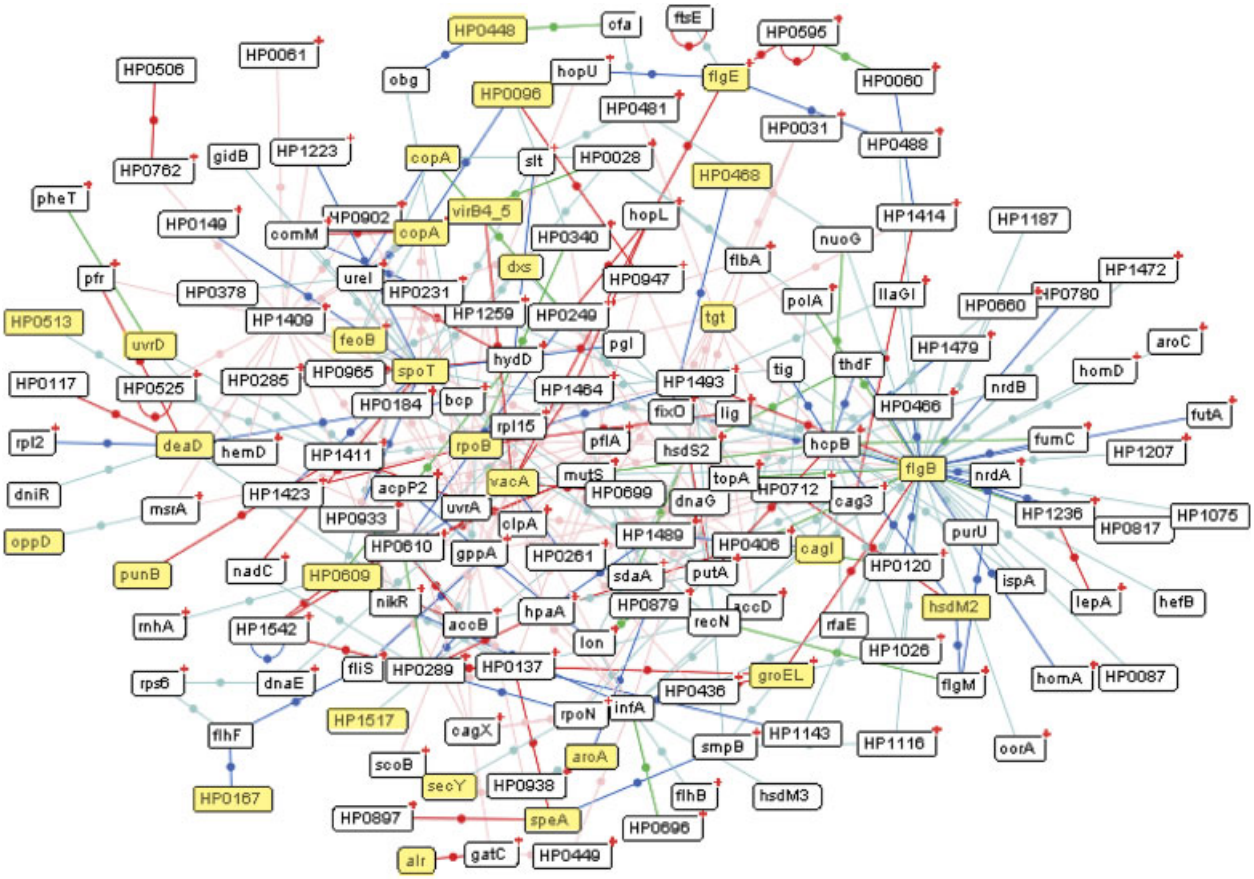

Figure 3. Interaction network of the identified phosphoproteins in *H. pylori*. Proteins in yellow represent the phosphoproteins identified in this study; proteins in gray are proteins interacted with the phosphoproteins. Interactions with different levels of reliability are assigned with different colours corresponding to PBS scores ranging from 0 to <1. The reliability decrease with the following sequence: red, blue, green, cyan and pink. A small red cross "+" is drawn on the top right of the protein whenever a protein contains partners that are not currently displayed within the map.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

GroEL and FlgB was previously observed in *B. subtilis* [55], albeit without any assignment of the in vivo function for such interaction. An earlier report discovered the molecular mimicry between a 41-kDa flagellar protein in *Borrelia burgdorferi* and HSP60, as the monoclonal antibody specific for the flagellar protein cross-reacted with the chaperonin protein [56]. However, it is not clarified whether the cross-reaction is due to the high-sequence similarity or the binding between the two proteins. *H. pylori* produces Flg to fulfill its requirement for the bacterial colonization of the human gastric mucosa. As a member in the HSP60 family, *H. pylori* GroEL has been shown to increase the risk of gastric carcinoma [57]. The previous studies have also demonstrated that the phosphorylation and dephosphorylation of GroEL regulated its binding and dissociation from unfolded proteins [58], possibly through the switch between the oligomeric states mediated by phosphorylation [59]. Further experimental procedures are needed to reveal the contribution of the GroEL and FlgB partners to the pathology of the bacterium with regard to how the phosphorylation of these two proteins regulates the bacterial colonization.

4 Concluding remarks

In this study, we globally characterized the phosphoproteome from the pathogenic bacterium *H. pylori*. Eighty-two phosphopeptides with 126 phosphorylation sites from 67 proteins were identified. The *H. pylori* phosphoproteome is characterized by in a comparably large phosphoproteome size, high abundance of Tyr and multiple phosphorylation and over-representation of membrane proteins. The GO analysis of the identified phosphoproteins indicated that *H. pylori* phosphorylation regulates enzymatic or catalytic activity, molecular interactions, molecule transporter and structural component, reflecting the close correlation between high level of phosphorylation and bacterial virulence and pathogenesis. An interaction network covering 28 phosphoproteins was constructed with a total of 163 proteins centering on the major *H. pylori* virulence factor VacA. The study presented here may provide the key clues for the novel targets in protein phosphorylation pathways, valuable for the discovery of new effective antibiotic against *H. pylori*.

This work was partially supported by the 2007 Chang-Jiang Scholars Program (to Q.-Y. H.), National Natural Science Foundation of China (20871057, to Q.-Y. H.; 20801061, to R. G.), Guangdong Natural Science Foundation (8451027501001233, to R. G.), Talents Start-up Foundation of Jinan University (JNU 51208047, to X. S.), SRF for ROCS, SEM (to R. G.) and the Fundamental Research Funds for the Central Universities (to R. G., X. S. and Q.-Y. H.).

The authors have declared no conflict of interest.

5 References

[1] Johnson, L. N., The regulation of protein phosphorylation. *Biochem. Soc. Trans.* 2009, *37*, 627–641.

[2] Cohen, P., The regulation of protein function by multisite phosphorylation a 25 year update. *Trends Biochem. Sci.* 2000, *25*, 596–601.

[3] Mann, M., Ong, S. E., Gronborg, M., Steen, H. et al., Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends Biotechnol.* 2002, *20*, 261–268.

[4] Ge, R., Sun, H., Metallomics: an integrated biometal science. *Sci. China Ser. B Chem.* 2009, *52*, 2055–2070.

[5] Paradela, A., Albar, J. P., Advances in the analysis of protein phosphorylation. *J. Proteome Res.* 2008, *7*, 1809–1818.

[6] Li, X., Gerber, S. A., Rudner, A. D., Beausoleil, S. A. et al., Large-scale phosphorylation analysis of alpha-factor-arrested *Saccharomyces cerevisiae*. *J. Proteome Res.* 2007, *6*, 1190–1197.

[7] Marshall, B. J., Warren, J. R., Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984, *1*, 1311–1315.

[8] Blaser, M. J., Ecology of *Helicobacter pylori* in the human stomach. *J. Clin. Invest.* 1997, *100*, 759–762.

[9] Cover, T. L., The vacuolating cytotoxin of *Helicobacter pylori*. *Mol. Microbiol.* 1996, *20*, 241–246.

[10] Backert, S., Selbach, M., Role of type IV secretion in *Helicobacter pylori* pathogenesis. *Cell. Microbiol.* 2008, *10*, 1573–1581.

[11] Salama, N. R., Otto, G., Tompkins, L., Falkow, S., Vacuolating cytotoxin of *Helicobacter pylori* plays a role during colonization in a mouse model of infection. *Infect. Immun.* 2001, *69*, 730–736.

[12] Costa, A. C., Figueiredo, C., Touati, E., Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* 2009, *14*, 15–20.

[13] Hatakeyama, M., Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. *Nat. Rev. Cancer* 2004, *4*, 688–694.

[14] Wen, S., Moss, S. F., *Helicobacter pylori* virulence factors in gastric carcinogenesis. *Cancer Lett.* 2009, *282*, 1–8.

[15] Shi, L., Potts, M., Kennelly, P. J., The serine, threonine, and/or tyrosine-specific protein kinases and protein phosphatases of prokaryotic organisms: a family portrait. *FEMS Microbiol. Rev.* 1998, *22*, 229–253.

[16] Grangeasse, C., Pichon, B., Bollen, A., Godfroid, E., Protein kinase activity in *Helicobacter pylori*. *FEMS Microbiol. Lett.* 1999, *176*, 327–332.

[17] Voisin, S., Watson, D. C., Tessier, L., Ding, W. et al., The cytoplasmic phosphoproteome of the Gram-negative bacterium *Campylobacter jejuni*: evidence for modification by unidentified protein kinases. *Proteomics* 2007, *7*, 4338–4348.

[18] Ge, R., Watt, R. M., Sun, X., Tanner, J. A. et al., Expression and characterization of a histidine-rich protein, Hpn: potential for Ni^{2+} storage in *Helicobacter pylori*. *Biochem. J.* 2006, *393*, 285–293.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[19] Sun, X., Ge, F., Xiao, C. L., Yin, X. F. et al., Phosphoproteomic analysis reveals the multiple roles of phosphorylation in pathogenic bacterium *Streptococcus pneumoniae*. *J. Proteome Res.* 2010, *9*, 275–282.

[20] Haas, W., Faherty, B. K., Gerber, S. A., Elias, J. E. et al., Optimization and use of peptide mass measurement accuracy in shotgun proteomics. *Mol. Cell. Proteomics* 2006, *5*, 1326–1337.

[21] Villen, J., Beausoleil, S. A., Gygi, S. P., Evaluation of the utility of neutral-loss-dependent MS3 strategies in large-scale phosphorylation analysis. *Proteomics* 2008, *8*, 4444–4452.

[22] Peng, J., Elias, J. E., Thoreen, C. C., Licklider, L. J., Gygi, S. P., Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. *J. Proteome Res.* 2003, *2*, 43–50.

[23] Macek, B., Gnad, F., Soufi, B., Kumar, C. et al., Phosphoproteome analysis of *E. coli* reveals evolutionary conservation of bacterial Ser/Thr/Tyr phosphorylation. *Mol. Cell. Proteomics* 2008, *7*, 299–307.

[24] Olsen, J. V., Blagoev, B., Gnad, F., Macek, B. et al., Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 2006, *127*, 635–648.

[25] Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J. et al., Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 2005, *21*, 3674–3676.

[26] Gardy, J. L., Spencer, C., Wang, K., Ester, M. et al., PSORT-B: Improving protein subcellular localization prediction for Gram-negative bacteria. *Nucleic Acids Res.* 2003, *31*, 3613–3617.

[27] Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A. et al., The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997, *388*, 539–547.

[28] Rain, J. C., Selig, L., De Reuse, H., Battaglia, V. et al., The protein-protein interaction map of *Helicobacter pylori*. *Nature* 2001, *409*, 211–215.

[29] Bradshaw, R. A., Burlingame, A. L., Carr, S., Aebersold, R., Reporting protein identification data: the next generation of guidelines. *Mol. Cell. Proteomics* 2006, *5*, 787–788.

[30] Parker, J. L., Jones, A. M., Serazetdinova, L., Saalbach, G. et al., Analysis of the phosphoproteome of the multicellular bacterium *Streptomyces coelicolor* A3(2) by protein/peptide fractionation, phosphopeptide enrichment and high-accuracy mass spectrometry. *Proteomics* 2010, *10*, 2486–2497.

[31] Lin, M. H., Hsu, T. L., Lin, S. Y., Pan, Y. J. et al., Phosphoproteomics of *Klebsiella pneumoniae* NTUH-K2044 reveals a tight link between tyrosine phosphorylation and virulence. *Mol. Cell. Proteomics* 2009, *8*, 2613–2623.

[32] Ravichandran, A., Sugiyama, N., Tomita, M., Swarup, S., Ishihama, Y., Ser/Thr/Tyr phosphoproteome analysis of pathogenic and non-pathogenic *Pseudomonas* species. *Proteomics* 2009, *9*, 2764–2775.

[33] Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J. et al., Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 1997, *91*, 511–520.

[34] Segal, E. D., Cha, J., Lo, J., Falkow, S., Tompkins, L. S., Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA* 1999, *96*, 14559–14564.

[35] Clifton, D. R., Fields, K. A., Grieshaber, S. S., Dooley, C. A. et al., A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc. Natl. Acad. Sci. USA* 2004, *101*, 10166–10171.

[36] Schulein, R., Guye, P., Rhomberg, T. A., Schmid, M. C. et al., A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells. *Proc. Natl. Acad. Sci. USA* 2005, *102*, 856–861.

[37] Cozzone, A. J., Bacterial tyrosine kinases: novel targets for antibacterial therapy? *Trends Microbiol.* 2009, *17*, 536–543.

[38] Mazmanian, S. K., Kasper, D. L., The love-hate relationship between bacterial polysaccharides and the host immune system. *Nat. Rev. Immunol.* 2006, *6*, 849–858.

[39] Grangeasse, C., Cozzone, A. J., Deutscher, J., Mijakovic, I., Tyrosine phosphorylation: an emerging regulatory device of bacterial physiology. *Trends Biochem. Sci.* 2007, *32*, 86–94.

[40] Jadeau, F., Bechet, E., Cozzone, A. J., Deleage, G. et al., Identification of the idiosyncratic bacterial protein tyrosine kinase (BY-kinase) family signature. *Bioinformatics* 2008, *24*, 2427–2430.

[41] Selbach, M., Moese, S., Backert, S., Jungblut, P. R., Meyer, T. F., The *Helicobacter pylori* CagA protein induces tyrosine dephosphorylation of ezrin. *Proteomics* 2004, *4*, 2961–2968.

[42] Bayle, D., Wangler, S., Weitzenegger, T., Steinhilber, W. et al., Properties of the P-type ATPases encoded by the copAP operons of *Helicobacter pylori* and *Helicobacter felis*. *J. Bacteriol.* 1998, *180*, 317–329.

[43] Rosen, R., Becher, D., Buttner, K., Biran, D. et al., Highly phosphorylated bacterial proteins. *Proteomics* 2004, *4*, 3068–3077.

[44] Soufi, B., Gnad, F., Jensen, P. R., Petranovic, D. et al., The Ser/Thr/Tyr phosphoproteome of *Lactococcus lactis* IL1403 reveals multiply phosphorylated proteins. *Proteomics* 2008, *8*, 3486–3493.

[45] Jers, C., Soufi, B., Grangeasse, C., Deutscher, J., Mijakovic, I., Phosphoproteomics in bacteria: towards a systemic understanding of bacterial phosphorylation networks. *Expert Rev. Proteomics* 2008, *5*, 619–627.

[46] Ge, R., Sun, X., Gu, Q., Watt, R. M. et al., A proteomic approach for the identification of bismuth-binding proteins in *Helicobacter pylori*. *J. Biol. Inorg. Chem.* 2007, *12*, 831–842.

[47] Sun, X., Ge, R., Chiu, J. F., Sun, H., He, Q. Y., Identification of proteins related to nickel homeostasis in *Helicobater pylori* by immobilized metal affinity chromatography and two-dimensional gel electrophoresis. *Metal Based Drugs* 2008, *2008*, 289490.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[48] Zhu, K., Zhao, J., Lubman, D. M., Miller, F. R., Barder, T. J., Protein pI shifts due to posttranslational modifications in the separation and characterization of proteins. *Anal. Chem.* 2005, *77*, 2745–2755.

[49] Gnad, F., Ren, S., Cox, J., Olsen, J. V. et al., PHOSIDA (phosphorylation site database): management, structural and evolutionary investigation, and prediction of phosphosites. *Genome Biol.* 2007, *8*, R250.

[50] Macek, B., Mijakovic, I., Olsen, J. V., Gnad, F. et al., The serine/threonine/tyrosine phosphoproteome of the model bacterium *Bacillus subtilis*. *Mol. Cell. Proteomics* 2007, *6*, 697–707.

[51] Miller, M. L., Soufi, B., Jers, C., Blom, N. et al., NetPhosBac – a predictor for Ser/Thr phosphorylation sites in bacterial proteins. *Proteomics* 2009, *9*, 116–125.

[52] Telford, J. L., Ghiara, P., Dell'Orco, M., Comanducci, M. et al., Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J. Exp. Med.* 1994, *179*, 1653–1658.

[53] Cover, T. L., Blanke, S. R., *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. *Nat. Rev. Microbiol.* 2005, *3*, 320–332.

[54] Ilver, D., Barone, S., Mercati, D., Lupetti, P., Telford, J. L., *Helicobacter pylori* toxin VacA is transferred to host cells via a novel contact-dependent mechanism. *Cell. Microbiol.* 2004, *6*, 167–174.

[55] Endo, A., Kurusu, Y., Identification of *in vivo* substrates of the chaperonin GroEL from *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.* 2007, *71*, 1073–1077.

[56] Dai, Z., Lackland, H., Stein, S., Li, Q. et al., Molecular mimicry in Lyme disease: monoclonal antibody H9724 to *B. burgdorferi* flagellin specifically detects chaperonin-HSP60. *Biochim. Biophys. Acta* 1993, *1181*, 97–100.

[57] Suerbaum, S., Thiberge, J. M., Kansau, I., Ferrero, R. L., Labigne, A., *Helicobacter pylori hspA-hspB* heat-shock gene cluster: nucleotide sequence, expression, putative function and immunogenicity. *Mol. Microbiol.* 1994, *14*, 959–974.

[58] Sherman, M., Goldberg, A. L., Heat shock-induced phosphorylation of GroEL alters its binding and dissociation from unfolded proteins. *J. Biol. Chem.* 1994, *269*, 31479–31483.

[59] Kumar, C. M., Khare, G., Srikanth, C. V., Tyagi, A. K. et al., Facilitated oligomerization of mycobacterial GroEL: evidence for phosphorylation-mediated oligomerization. *J. Bacteriol.* 2009, *191*, 6525–6538.

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim