The Serine/Threonine/Tyrosine Phosphoproteome of the Model Bacterium *Bacillus subtilis**

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Protein phosphorylation on serine, threonine, and tyrosine (Ser/Thr/Tyr) is well established as a key regulatory post-translational modification in eukaryotes, but little is known about its extent and function in prokaryotes. Although protein kinases and phosphatases have been predicted and identified in a variety of bacterial species, classical biochemical approaches have so far revealed only a few substrate proteins and even fewer phosphorylation sites. *Bacillus subtilis* is a model Gram-positive bacterium in which two-dimensional electrophoresis-based studies suggest that the Ser/Thr/Tyr phosphorylation should be present on more than a hundred proteins. However, so far only 16 phosphorylation sites on eight of its proteins have been determined, mostly in *in vitro* studies. Here we performed a global, gel-free, and site-specific analysis of the *B. subtilis* phosphoproteome using high accuracy mass spectrometry in combination with biochemical enrichment of phosphopeptides from digested cell lysates. We identified 103 unique phosphopeptides from 78 *B. subtilis* proteins and determined 78 phosphorylation sites: 54 on serine, 16 on threonine, and eight on tyrosine. Detected phosphoproteins are involved in a wide variety of metabolic processes but are enriched in carbohydrate metabolism. We report phosphorylation sites on almost all glycolytic and tricarboxylic acid cycle enzymes, several kinases, and members of the phosphoenolpyruvate-dependent phosphotransferase system. This significantly enlarged number of bacterial proteins known to be phosphorylated on Ser/Thr/Tyr residues strongly supports the emerging view that protein phosphorylation is a general and fundamental regulatory process, not restricted only to eukaryotes, and opens the way for its detailed functional analysis in bacteria. ***Molecular & Cellular Proteomics 6:697–707, 2007.***

Protein phosphorylation is a reversible post-translational modification that has tremendous regulatory and signaling potential (1). Eukaryotic cells rely extensively on phosphorylating the hydroxyl group of the side chains of serine/threonine/tyrosine for their signal transduction cascades (2, 3). The importance of serine/threonine/tyrosine kinases and phosphatases for cell physiology has been widely documented in eukaryotes ranging from yeast (4) to human (5).

Protein phosphorylation in bacteria is less intensely studied. Initial studies have revealed that bacteria use histidine/aspartate phosphorylation, mainly in their two-component systems, which represent a paradigm of bacterial signal transduction (6). Histidine phosphorylation in bacteria also takes place in the phosphoenolpyruvate-dependent phosphotransferase systems (PTSs),[1] which are involved in internalization and phosphorylation of sugars (7). Several participants of the PTS phosphorelay, such as HPr and enzyme IIA, also participate in regulatory events that depend on their own level of phosphorylation (8).

For some time the field of protein phosphorylation maintained this clear-cut picture of eukaryotes using serine/threonine/tyrosine phosphorylation and bacteria using histidine and aspartate phosphorylation instead. However, in the last 2 decades evidence has accumulated for serine/threonine/tyrosine phosphorylation in bacteria (9). Bacteria possess kinases and phosphatases that structurally resemble their eukaryotic counterparts (10) but have also developed idiosyncratic kinases and phosphatases with no known homologues in eukaryotes. Bacterial tyrosine kinases with Walker motifs A, A′, and B are a good example of such strictly bacterial kinases (11). Interestingly signaling via serine/threonine/tyrosine phosphorylation is often implicated in the regulation of bacterial virulence (12), and in some cases it is also known to interfere with eukaryotic signal transduction, thereby rendering the host more prone to infection (13, 14).

A long tradition of fundamental research on *Bacillus subtilis*, a model Gram-positive bacterium, has contributed to deciphering complex physiological and regulatory phenomena such as sporulation, natural competence, and carbon catab-

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[1] The abbreviations used are: PTS, phosphoenolpyruvate-dependent phosphotransferase system; 2D, two-dimensional; SCX, strong cation exchange; LB, Luria-Bertani; GO, gene ontology; SIM, selected ion monitoring; LTQ, linear ion trap; *R*, resolution; HCD, higher energy collision-induced dissociation.

olite regulation. *B. subtilis* is also of significant industrial importance as it is often used as a cell factory for enzyme production. Among bacteria, this organism stands out as the one with intensively studied protein phosphorylation and the one with several identified tyrosine-phosphorylated proteins (11, 15). However, so far phosphorylation sites on serine, threonine, or tyrosine have been determined in only eight of its proteins according to the prokaryotic Phosphorylation Site Database (16).

In the past, global approaches to analyze bacterial phosphoproteomes relied exclusively on 2D gels followed by mass spectrometric identification of radiolabeled spots. Such studies were performed in both Gram-negative and Gram-positive bacteria, including *B. subtilis*, and have revealed several dozen proteins phosphorylated at serine/threonine residues but no tyrosine-phosphorylated proteins and no phosphorylation sites (17–20). Instead all identified bacterial phosphotyrosine residues come from individual *in vitro* biochemical studies, which detected a number of autophosphorylating tyrosine kinases (21–23) and their protein substrates (24–27), and the same is true for phosphoserine and phosphothreonine residues (28–35).

In the last few years peptide-based phosphoproteomics by mass spectrometry, instead of 2D gel electrophoresis, has become increasingly powerful (36–38). It is now possible to determine hundreds or even thousands of phosphorylation sites in a single investigation (39–41). In this study we enzymatically digested the entire *B. subtilis* proteome and enriched phosphopeptides by titanium oxide chromatography (42). Phosphopeptides were measured in a hybrid linear ion trap-Fourier transform ion cyclotron resonance or an orbitrap mass spectrometer, which determines their mass and fragmentation spectra. This gel-free approach has recently proven to be extremely powerful in studies of eukaryotic protein phosphorylation and allowed the determination of more than 6600 phosphorylation sites in HeLa cells (43). Here we used this technology to obtain a site-specific, *in vivo* phosphoproteome of *B. subtilis*.

EXPERIMENTAL PROCEDURES

Cell Culture and Lysis—Wild type *B. subtilis* cells (strain 168) were grown in 12 liters of LB medium under vigorous shaking. At $A_{600} = 1$ the cells were precipitated by centrifugation (5 min at 600 × *g*) and resuspended in lysis buffer containing 50 mM Tris-Cl (pH 7.5), 5 mg/ml lysozyme, and a 5 mM concentration of each of the following phosphatase inhibitors: sodium fluoride, 2-glycerol phosphate, sodium vanadate, and sodium pyrophosphate (Sigma). Cell wall lysis was performed for 15 min at 37 °C, and cell membranes were disrupted by sonication. DNase I (100 μg/ml) (Sigma) was added to the lysate and incubated for an additional 10 min at 37 °C. *N*-Octyl glucoside detergent (Sigma) was added to a final concentration of 1% for more efficient extraction and solubilization of membrane proteins. Cellular debris were removed by centrifugation at 25,000 × *g* for 30 min. The crude protein extract was extensively dialyzed against deionized water and finally lyophilized.

Protein Digestion and Phosphopeptide Enrichment—Protein concentration in the crude protein extract was estimated by Bradford reagent (Bio-Rad). About 10 mg of protein extract was dissolved and denatured in 6 M urea and 2 M thiourea, reduced with 1 mM DTT for 45 min at room temperature, and carbamidomethylated with 5 mM iodoacetamide for 45 min at room temperature in the dark. Alkylated proteins were digested first with endopeptidase Lys-C (Waco) for 3 h, after which the solution was diluted four times with deionized water, and then further digested with sequencing grade modified trypsin (Promega) overnight. The protease/protein ratio was in both cases 1:50. The resulting peptide mixture was acidified with TFA to pH <3 and subjected to two stages of phosphopeptide enrichment.

In the first stage, strong cation exchange chromatography was performed as described previously (40) with minor modifications. Samples were loaded onto a 1-ml Resource S column (GE Healthcare) in solvent A (5 mM KH_2PO_4, 30% acetonitrile, 0.1% trifluoroacetic acid, pH = 2.7) at a flow rate of 1 ml/min. Elution was performed with a gradient of 0–30% solvent B (5 mM KH_2PO_4, 30% acetonitrile, 350 mM KCl, 0.1% trifluoroacetic acid, pH = 2.7) over 30 min. Fifteen 2-ml fractions as well as the flow-through were collected and subjected separately to the second stage of phosphopeptide enrichment.

The second stage of phosphopeptide enrichment was performed using titanium dioxide (TiO_2) chromatography (42, 43). The TiO_2 beads (kindly provided by GL Sciences, Tokyo, Japan), and all strong cation exchange (SCX) fractions were preincubated with 5 mg/ml 2,5-dihydroxybenzoic acid in 80% acetonitrile. Each SCX fraction was then added to a 2-ml reaction tube containing ~10 mg of the TiO_2 beads and incubated batchwise with end-over-end rotation for 30 min. After incubation, the beads were spun down and washed two times with acetonitrile/water (1:1) solution containing 0.2% trifluoroacetic acid. Bound peptides were eluted from the column with 0.5% ammonium solution, pH 10.5, in 40% acetonitrile; dried almost to completeness; and reconstituted in 1% TFA, 2% acetonitrile in water for LC-MS analysis.

Liquid Chromatography-Mass Spectrometry—Liquid chromatography was performed on a 1100 nano-HPLC system (Agilent Technologies) fitted with an in-house made 75-μm reverse phase C_{18} column as described previously (44). Each sample was loaded in solvent A (0.5% acetic acid in water) and eluted with a segmented gradient of 10–60% solvent B (80% acetonitrile, 0.5% acetic acid in water) over 120 min. The HPLC system was coupled to either an LTQ-FT or LTQ-Orbitrap mass spectrometer (Thermo Electron) via a nanoscale LC interface (Proxeon Biosystems).

In the LTQ-FT mass spectrometer samples were measured in duplicate; in the first measurement the survey scan was performed in the FT-ICR analyzer and followed by SIM scans of the three most intense peptide ions at resolution (*R*) of 50,000 and their MS^2 fragmentation in the linear ion trap (LTQ) ("FT-SIM" method) (45). The second LTQ-FT measurement was performed by the survey scan acquisition in the FT-ICR analyzer (at $R = 100{,}000$) followed by MS^2 of the five most intense peptide ions in the LTQ and MS^3 of all ions showing the neutral loss of phosphoric acid (98 Da) from the precursor ion as described previously (40) ("FT-Top5" method).

In the LTQ-Orbitrap mass spectrometer samples were measured in triplicate. In the first measurement the survey scan was performed in the orbitrap analyzer (at $R = 60{,}000$) and was followed by MS^2 of the three most intense ions in both the orbitrap (at $R = 15{,}000$) and the LTQ analyzers ("Orbitrap-FT" method). The second measurement was performed by the survey scan acquisition in the orbitrap (at $R = 30{,}000$) and subsequent MS^2 in both the C-trap (acquired in the orbitrap at $R = 15{,}000$) and the LTQ ("Orbitrap HCD" method). The final measurement was performed by the survey scan acquisition in the orbitrap (at $R = 60{,}000$) and MS^2 of the five most intense ions in the LTQ ("Orbitrap-Top5" method). All measurements in the orbitrap mass analyzer were performed with on-the-fly internal recalibration by "locking" to polydimethylcyclosiloxane ions at *m/z* 445.120025 and

429.088735, as described previously (46), for further improvement of mass accuracy.

Data Processing, Validation, and Analysis—Peak lists for the database search were produced in the Mascot generic format using the in-house developed software DTASuperCharge (SourceForge) for the LTQ-FT spectra and Raw2msm (46) for the LTQ-Orbitrap spectra. Because bacteria were grown on the LB medium containing yeast extract, a concatenated database consisting of forward and reversed sequences of the *B. subtilis* strain 168 (forward protein database downloaded from cmr.tigr.org), *Saccharomyces cerevisiae* (yeast_orf database, *Saccharomyces* Genome Database, Stanford University), and 26 most commonly observed contaminants in MS measurements was created and searched using the Mascot search engine (Matrix Science). The search criteria were as follows: full tryptic specificity was required; two missed cleavages were allowed; carbamidomethylation was set as fixed modification; oxidation (Met), *N*-acetylation (protein), and phosphorylation (STY, His, and Asp) were set as variable modifications; precursor ion mass tolerances were 10 ppm for all measurements; fragment ion mass tolerance was 0.5 Da for all MS^2 spectra acquired in the LTQ and 0.02 Da for all MS^2 spectra acquired in the orbitrap mass analyzer.

Mass spectra of identified phosphopeptides were analyzed using the MSQuant software (SourceForge). All spectra were manually validated, and the following acceptance criteria were applied: precursor ion mass tolerance required for all orbitrap and FT-SIM measurements was 5 ppm and for FT-Top5 was 10 ppm. All Ser(P) and Thr(P) peptides were required to show a pronounced neutral loss of phosphoric acid from the precursor ion and/or fragment ions or trigger the neutral loss-dependant MS^3 scan. Only peptides containing more than 6 amino acid residues were considered, and extensive coverage of b- and/or y-ion series was required. All proline-containing peptides were required to show pronounced cleavage N-terminally to the Pro residue. Phosphopeptides that did not show this typical fragmentation pattern were accepted only if they were detected in two or more different measurements or in two or more forms (*e.g.* with or without methionine oxidation or as a complete and a missed cleavage). In phosphopeptides with multiple potential phosphorylation sites, the probabilities for phosphorylation at each site were calculated from the post-translational modification scores as described previously (43).

Bioinformatics Analysis—Information on function and localization of identified phosphoproteins was extracted from the Swiss-Prot database. To gain information on the over-representation of certain protein classes among the identified phosphoproteins, enrichment analysis of their gene ontology (GO) terms was performed. Because there is no GO annotation available for the *B. subtilis* proteome, putative GO terms were assigned using the Blast2GO tool (47). Blast2GO assigned the GO annotation to a protein with unknown function based on its sequence similarity to other proteins in Swiss-Prot. Using this tool we could annotate 1298 *B. subtilis* proteins known to be expressed in the exponential growth phase to 1281 distinct GO terms, which were used as the reference dataset; the test dataset for enrichment analysis was the list of phosphoproteins identified in this study. To find statistically over-represented GO categories among phosphoproteins identified in this study, the BiNGO-Cytoscape plugin (48) was used. The annotations for the reference dataset were converted to a custom ontology as instructed on the BiNGO webpage. The enrichment analysis was done using "Hyper-Geometric test" with correction for multiple hypothesis testing, and all GO terms that were significant with $p < 0.01$ were selected as over-represented.

RESULTS

In this global, label-free, and gel-free study of the *B. subtilis* phosphoproteome, cells growing in the commonly used LB medium were lysed, and their proteins were extracted, denatured, and digested with endoproteases Lys-C and trypsin in solution. Phosphopeptides were separated and enriched from the resulting complex peptide mixtures using SCX and TiO_2 chromatographies. They were then analyzed using nanoscale LC coupled to high resolution hybrid mass spectrometers (LTQ-FT and LTQ-Orbitrap). Peptides were subjected to two subsequent stages of fragmentation in the linear ion trap part of the instrument, while the high resolution analyzer simultaneously acquired the precise peptide masses. To increase phosphopeptide coverage and confidence of identification, samples were analyzed multiple times using different acquisition strategies. This proved to be very important because it increased the chance of sampling low abundance phosphopeptides. Stringent validation criteria for each of the identified peptides, especially in terms of the precursor ion mass accuracy and fragmentation pattern in MS^2 and MS^3 measurements, were applied to maximize the confidence of identification. Absolute average mass accuracies and standard deviations in different measurements were as follows: FT-SIM, 1.2/0.75 ppm; FT-Top5, 3.98/2.45 ppm; Orbitrap-FT, 0.85/0.76 ppm; Orbitrap-HCD, 1.13/1.26 ppm; and Orbitrap-Top5, 1.01/1.26 ppm (see "Experimental Procedures" for a description of the measurements). In accordance with standards for the identification of phosphorylation sites (49), a spectrum for each of the phosphopeptide assignments is listed in the supplemental material. For phosphopeptides with multiple potential phosphorylation sites we calculated a probability score for each potential phosphorylation site (43).

We also attempted to enrich for tyrosine-phosphorylated proteins. However, immunoprecipitation with anti-phosphotyrosine antibody only yielded one additional tyrosine phosphorylation site.

Phosphoproteome of Exponentially Growing B. subtilis—Our approach resulted in the identification of 103 unique phosphopeptides from 78 *B. subtilis* proteins with very high confidence. In the majority of the cases (78 of 103) the site of phosphorylation in the identified phosphopeptide was furthermore determined with a probability higher than 75%. In the other 24 phosphopeptides the modification sites could not be unambiguously determined from the mass spectra, but they are still localized to the short amino acid sequences in the phosphopeptides. All phosphopeptides and phosphorylation sites are presented in Table I. Detailed information relevant to identified phosphopeptides is listed in Supplemental Table 1 and can also be accessed in the PHOSIDA database (www.phosida.com) (43).

Among the identified phosphorylation sites, 54 (69.2%) were on serine, 16 (20.5%) were on threonine, and eight (10.3%) were on tyrosine. Although the dataset was searched against the *B. subtilis* protein database considering histidine and aspartate phosphorylation, no compelling evidence for the presence of these modifications was found. This was expected because the experimental conditions were unfavor-

TABLE I

Phosphoproteins, phosphopeptides, and phosphorylation sites detected in this study

The non-redundant gene names, instead of proteins names, are used for clarity. Ambiguous phosphorylation sites are presented in brackets. Detailed information on detected phosphopeptides can be found in Supplemental Table 1.

Gene name	Phosphopeptide sequence	Gene name	Phosphopeptide sequence
ahpF	DMLALVDELASMp[SS]K	*rsbR*	SQVVLIDITGVPVVDp[TMVAHHIIQAS]EAVR
ahpF	RLp[YS]LPNVTVVK	*rsbR*	IALQELSAPLIPVFENITVMPLVGTIDpTERAK
aroA	pSNTELELLRQK	*rsbR*	IALQELSAPLIPVFENITVMPLVGTIDpTER
asd	RGAIVIDNTpSAFR	*rsbS*	IYETGANGVVIDLTSVDMIDpSFIAK
asd	KApYGLNK	*rsbV*	DVpSYMDSTGLGVFVGTFK
citH	ERVIGQpSGVLDTAR	*rsbV*	DVSYMDpSTGLGVFVGTFK
citZ	RLTNLTGEpSK	*rsbV*	DVSYMDSpTGLGVFVGTFK
codY	pSVIVNALRK	*sodA*	SVEELVADLDSVPENIRpTAVR
cysS	pSLGNFVLVHDIIK	*sodA*	HHNpTYVTNLNK
degS	NRLpSEVSR	*spoIIAA*	HIVLNLEDLSFMDp[SS]GLGVILGR
dhbC	TLEVPEKPpSLIK	*spoVG*	DITHPINp[SST]R
dhbF	VGIDDSFFELGGHpSLLAAR	*srfAA*	AGIFDNFFETGGHpSLK
drm	Dp[TMT]GHWEIMGLYIDKPFK	*srfAB*	AGVTDNFFMIGGHpSLK
eno	pTLPVPMMNIVNGGEHADNNVDIQEFMIMPVGAPNFR	*srfAB*	TLGIEAIGIDDNFFDLGGHpSLK
eno	YHLpSGEGVVK	*srfAC*	QIGIHDDFFALGGHpSLK
eno	KLpSEGIK	*sucC*	LNFDpSNALYR
eno	p[YPIIS]IEDGLDENDWEGHK	*sucD*	VIVQGITGp[ST]ALFHTK
fbaA	INVNpTENQISSAK	*tagE*	pSLHAVSESNIK
fbaA	STGLPLVLHGGpTGIPTADIKK	*tpi*	IQYGGpSVKPANIK
fus	LFDGpSYHDVDSNEMAFK	*tsf*	DDpSSAFGAYLHMGGR
fus	EQAEELRNpSLIEAVCELDEELMDK	*ybbI*	pSEPLNLHR
fus	IGEp[THEGAS]QMDWMEQEQER	*ybbT*	AMDAEAGVMISApSHNPVQDNGIK
fus	pSVAEEIIKK	*ycnE*	REEFLSEAQpSLVQHSR
fus	NIGIMAHIDAGKp[TT]TTER	*yerA*	GGLVPLELSYDMTMDDLQFpSMPMGVK
fus	HpSSDEEPFSALAFK	*yerB*	FLAIFQSQMPEp[TVGPVRS]AR
gap	YDAANHDVIp[SNASCTT]NCLAPFAK	*yfiY*	p[SGNAHEVDDVVWT]TAGGIK
glgP	p[THKS]LSGLHK	*yfkK*	ESFSPpSEMQAIAQELASLRK
hbs	MNKpTELINAVAEASELSK	*yfkK*	KESFSPpSEMQAIAQELASLRK
infA	VTVELSPpYDLTR	*yfkK*	KESFSPpSEMQAIAQELASLR
ispU	STALLPLVGDIDpTERAK	*yfnI*	p[TSDAELT]MDNSIFGLPEGSAFVTK
ispU	STALLPLVGDIDpTER	*yjdD*	RKFEApSQR
lctE	NAApYHIIEK	*yjoA*	EMGHTELPFpYQQR
licB	DYTIWAVpSGDSVQNHIDK	*yloP*	RFTIQEDEEMpTK
mtlA	SEVLHpSGISIIQIPEGVEYGEGNTAK	*ynfE*	MDEILKQYMVLpYKK
ndk	NIIHGSDSLEpSAER	*yojH*	DMITELSAPVIVLFHSVGLLPLIGDIDpTVRAK
ndk	QLIGKpTNPK	*yojH*	DMITELpSAPVIVLFHSVGLLPLIGDIDTVRAK
ndk	NIIHGp[SDSLES]AEREINIFFK	*yojH*	DMITELSAPVIVLFHSVGLLPLIGDIDpTVR
ndk	NIIHGSDp[SLES]AER	*yojH*	DMITELSAPVIVLFHpSVGLLPLIGDIDTVR
oppA	NGGNNDpTGWENPEFKK	*yorK*	EFILQLDGpYK
oppA	DGSLHVEPIAGVp[YWY]K	*ypfD*	VLpSVDRDNER
pdhB	VAAPDTVFPFp[SQAES]VWLPNHK	*ypoC*	pSYPAKPINWAER
pgi	pTGAGSDFLGWVDLPEHYDK	*ypsB*	KQPVQp[SNT]TNFDILK
pgk	AVpSNPDRPFTAIIGGAK	*yqbO*	SpSIpSETLFSK
pgk	pTRETYADVIK	*yqfN*	SGLNpSHISVR
pgm	YWNQYPHQTLTASGEAVGLPEGQMGNpSEVGHLNIGAGR	*yqhA*	EMINELSAPIMPITDGIGILPLVGEIDpTHRAR
pnbA	ENISAFGGDPDNVTVFGEpSAGGMSIAALLAMPAAK	*yqjI*	AGp[TAT]DATIQSLLPHLEK
pnp	IGLILGpSGLGILADEIENPVK	*ytnP*	FGVVPKPLWpSK
pta	GLADGLVSGAAHp[ST]ADTVRPALQIIK	*ytxJ*	WHp[TSHSQIT]EAAIEQHLS
ptsH	VTADpSGIHARPATVLVQTASK	*yvyG*	pYIQAITQTEDDRIK
ptsH	pSIMGVMSLGIAK	*ywfI*	LLp[SSDERQSII]HEFTGLLEK
ptsI	NIp[SDS]EAEVSRFDEAIAR	*ywjH*	EANVpSFHDR
pykA	LNFpSHGDFEEHGAR	*ywqF*	LNFETp[SY]EK
pyrB	RGEAAYVIpSH	*yxxG*	IIFDDp[YKES]K
rocA	p[TVTY]AHEPFTDFTEAK		

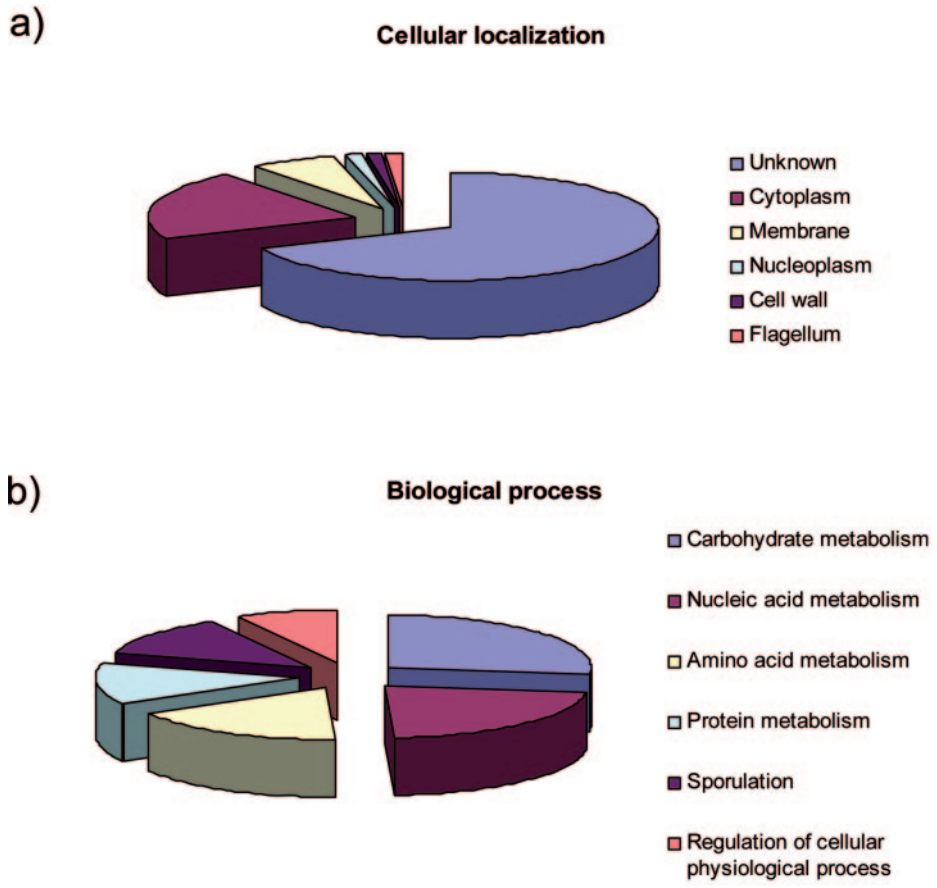

FIG. 1. **Cellular localization (*a*) and biological process (*b*) of the *B. subtilis* phosphoproteome as assigned by Blast2GO.** By sequence similarity, most of the proteins in the "unknown" category (not recognized by Blast2GO) are cytoplasmic.

able for detection of these modifications as they are unstable in acidic conditions, and at several stages of analysis peptides were exposed to low pH values (down to pH 1 during sample preparation for LC-MS).

Classes of Phosphorylated Proteins—We used the Blast2GO tool to extract the GO terms for *B. subtilis* proteins from their closest GO-annotated orthologs in the Swiss-Prot database. Of 78 phosphoproteins identified in this study, for 60 information on biological process was obtained, and for 26 information on cellular localization was obtained. Only 1305 of 4100 *B. subtilis* genes had a putative cellular component annotation, and the same pattern manifests in our annotation.

Phosphoproteins are present in all parts of the bacterial cell (Fig. 1*a*) and distribute among a wide variety of metabolic and regulatory enzymes that are normally expressed in the growth phase (Fig. 1*b*). Interestingly a high proportion of proteins with hitherto unknown functions was also found to be phosphorylated. GO enrichment analysis against the growth phase proteome of *B. subtilis* showed that protein phosphorylation is statistically over-represented among enzymes involved in the main pathways of carbohydrate metabolism ($p = 3.6 \times 10^{-7}$). Indeed we detected phosphorylation sites on almost all glycolytic enzymes, including enolase (*eno*), L-lactate dehydrogenase (*lctE*) (tyrosine-phosphorylated), triose-phosphate isomerase (*tpi*), glyceraldehyde-3-phosphate dehydrogenase (*gap*), pyruvate kinase (*pykA*), malate dehydrogenase (*citH*), phosphoglycerate mutase (*pgm*), glucose-6-phosphate isomerase (*pgi*), fructose-1,6-bisphosphate aldolase (*fbaA*), pyruvate dehydrogenase (*pdhB*), phosphoglycerate kinase (*pgk*), and "similar to phosphoglucomutase" (*ybbT*). In addition, phosphorylation was detected on several members of the tricarboxylic acid cycle, such as citrate synthase II (*citZ*) and succinyl-CoA synthetase (α and β subunits; *sucD* and *sucC*, respectively) and several members of the pentose phosphate pathway.

Among other phosphoproteins, several are involved in DNA metabolism and protein biosynthesis, such as nonspecific DNA-binding protein HBsu (*hbs*), aspartate-semialdehyde dehydrogenase (*asd*), cysteinyl-tRNA synthetase (*cysS*), initiation factor IF-I (*infA*) (tyrosine-phosphorylated; Fig. 2), and elongation factor Ts (*tsf*). These proteins, together with the glycolytic enzymes *eno*, *fbaA*, *pgk*, *pgm*, *tpi*, and *ybbT*, were recently classified as essential in *B. subtilis* (50).

Other regulatory proteins found to be phosphorylated are protein kinases such as the two-component sensor histidine kinase (*degS*) and several members of the PTS system: enzyme I (*ptsI*), histidine-containing phosphocarrier protein HPr (*ptsH*) (Fig. 3), mannitol-specific enzyme IIABC component

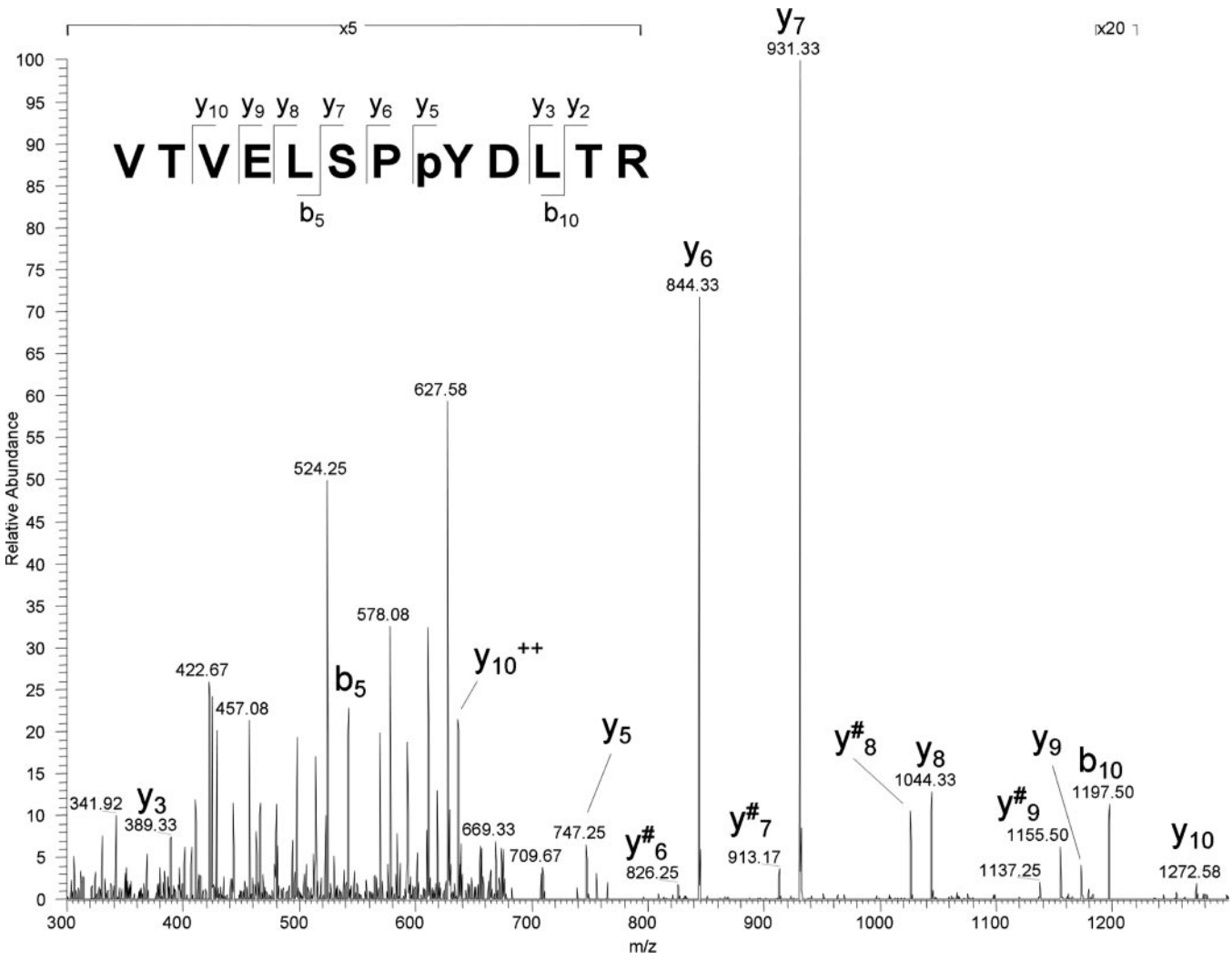

FIG. 2. **MS^2 spectrum of the tyrosine-phosphorylated peptide from initiation factor IF-I (*infA*).** Precursor ion mass was measured in the orbitrap mass spectrometer (mass deviation, 0.53 ppm), and the MS^2 spectrum was acquired in the LTQ mass spectrometer. Fragment ions arising from the loss of water are marked with #. *pY*, phosphotyrosine.

(*mtlA*), and lichenan-specific enzyme IIB component (*licB*). Importantly the global transcriptional regulator CodY is also a phosphoprotein (Fig. 4).

To investigate whether similarities exist between structural determinants for kinase action in bacteria and eukaryotes, we tested whether amino acid sequences surrounding the identified phosphorylation sites in *B. subtilis* resemble the target motifs of 15 common eukaryotic kinases. Although 17 Ser/Thr-phosphorylated peptide sequences matched the target motifs for eukaryotic casein kinases CK1 (S*XX*(pS/pT)) and (S/T)*XXX*pS where pS is phosphoserine and pT is phosphothreonine) and CK2 ((pS/pT)*XX*E), this distribution corresponded to expected frequencies of these motifs obtained by chance.

DISCUSSION

Comparison with Previous Studies of the B. subtilis Phosphoproteome—Phosphorylation on several *B. subtilis* proteins was previously the subject of targeted studies in which proteins were typically overexpressed, purified, *in vitro* phosphorylated, and analyzed by various classical biochemical methods sometimes combined with mass spectrometry (51). Of 16 phosphorylation sites from eight proteins reported so far, we detected seven sites on six proteins, all in agreement with those published previously. We found only one of eight phosphorylation sites on PrkC (gene name *yloP*), reported previously in a detailed *in vitro* mutagenesis study (31), and did not detect phosphorylation sites on SsbA and Idh. We note that our dataset represents the *in vivo* phosphoproteome because bacterial cells were grown in normal laboratory conditions and were not treated prior to harvesting. It is likely that some of the previously documented phosphorylation events occur only in specific circumstances, if at all, *in vivo*. For example, the tyrosine kinase PtkA (previously known as YwqD) was not identified in this study, although it was found

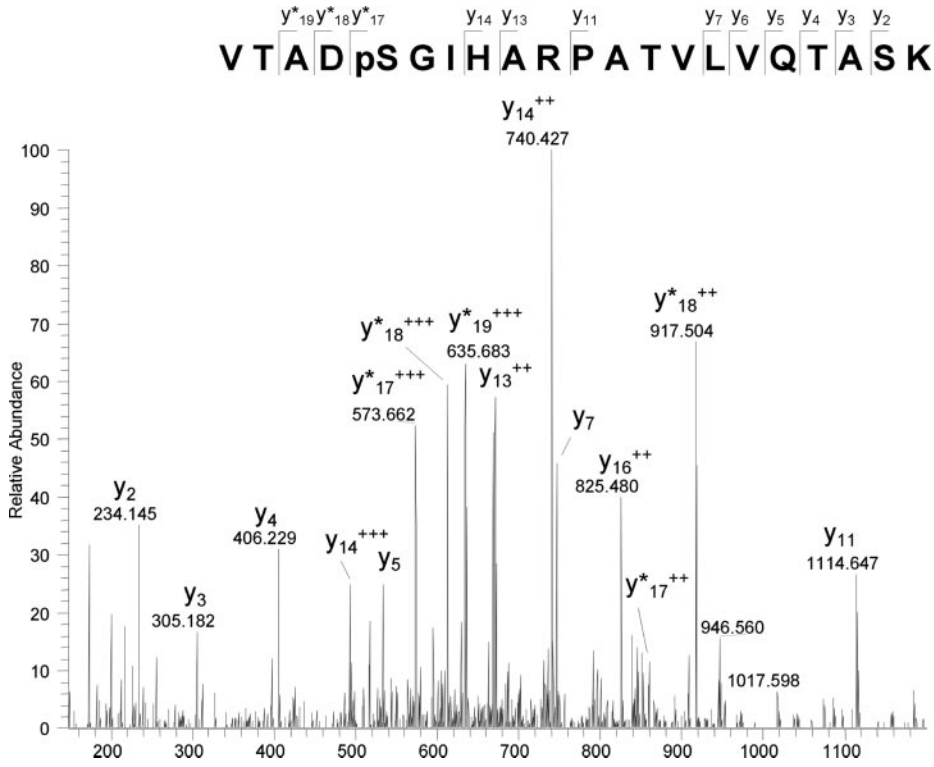

FIG. 3. **MS^2 spectrum of the serine-phosphorylated peptide from the histidine-containing phosphocarrier protein Hpr (gene name *ptsH*).** Precursor ion mass was measured in the orbitrap (mass deviation, 0.91 ppm); MS^2 was performed in the C-trap and measured in the orbitrap. No neutral loss of phosphate (−80) from the precursor ion or any evidence of modified y-ions containing histidine residue was observed, making histidine phosphorylation highly improbable. Instead pronounced neutral loss of phosphoric acid (−98) from the y-ion series starting from y_{17} (marked with *) points to serine as the modification site. *pS*, phosphoserine.

to autophosphorylate quite efficiently *in vitro*. By contrast, we identified a phosphorylated substrate of PtkA, Ugd (previously known as YwqF), although Ugd phosphorylation *in vitro* is much less efficient than that of PtkA (25).

The Ser/Thr/Tyr phosphoproteome of *B. subtilis* was recently the subject of a global study where cells were grown in a synthetic medium and radiolabeled with ^{32}P, and their protein extracts were analyzed using 2D gel electrophoresis (pI range, 4–7) and mass spectrometry (20). In that study 29 proteins were reported, however, without information on phosphorylation sites. 2D gels are limited by protein pI range and solubility; at least five proteins in our dataset have pI higher than 7, and an additional five are membrane proteins and are therefore inaccessible to 2D gels. Another potential problem in 2D gels is the fact that the identified protein detected after excision of the spot on the gel may be a co-migrating protein that was more abundant than the radioactively labeled one. In this regard it is notable that only five of the proteins detected in the 2D gel study are contained in our dataset.

Insights into the Bacterial Phosphoproteome—According to the prokaryotic Phosphorylation Site Database (16), Ser/Thr/Tyr phosphorylation has so far been reported in about 35 bacterial species, resulting in determination of about 70 phosphorylation sites. The dataset presented here is therefore the most comprehensive study of Ser/Thr/Tyr phosphorylation in a prokaryotic organism thus far and provides insight into the phosphoproteome of a model Gram-positive bacterium. A striking distinction to known metazoan phosphoproteomes is in its extent; whereas in humans more than 30% of proteins are phosphorylated, this and other studies (17, 20) show that this number is at least an order of magnitude lower in bacteria. This may reflect a difference in the primary role of Ser/Thr/Tyr in eukaryotes and prokaryotes; in the former it is extensively used for transduction of signals within and between the cells, whereas in the latter its function is probably less central.

Tyrosine phosphorylation in eukaryotes is usually thought to have arisen in connection with multicellularity, and accordingly metazoans have a higher proportion than unicellular eukaryotes such as *S. cerevisiae*, which has very few tyrosine sites. An interesting observation that emerges directly from our data is the relatively high extent of tyrosine phosphorylation. We found a ratio of 70:20:10 for Ser/Thr/Tyr in *B. subtilis*, more similar to metazoans than to yeast. For example, we have recently found this ratio to be 86:12:2 in human cell culture (43). However, to see how general this phenomenon is, more phosphorylation sites from different eubacteria would be needed. Conversely our finding that bacterial phosphorylation sites do not match any of the common target sequences for eukaryotic kinases, together with the fact that *B. subtilis* possesses kinases without good eukaryotic orthologs, suggests that Ser/Thr/Tyr phosphorylation is not simply adapted from eukaryotes via horizontal gene transfer.

Ser/Thr/Tyr Phosphorylation in Metabolic Pathways—Phosphoproteins detected in this study participate in a wide variety of metabolic pathways, including metabolism of nucleic acids,

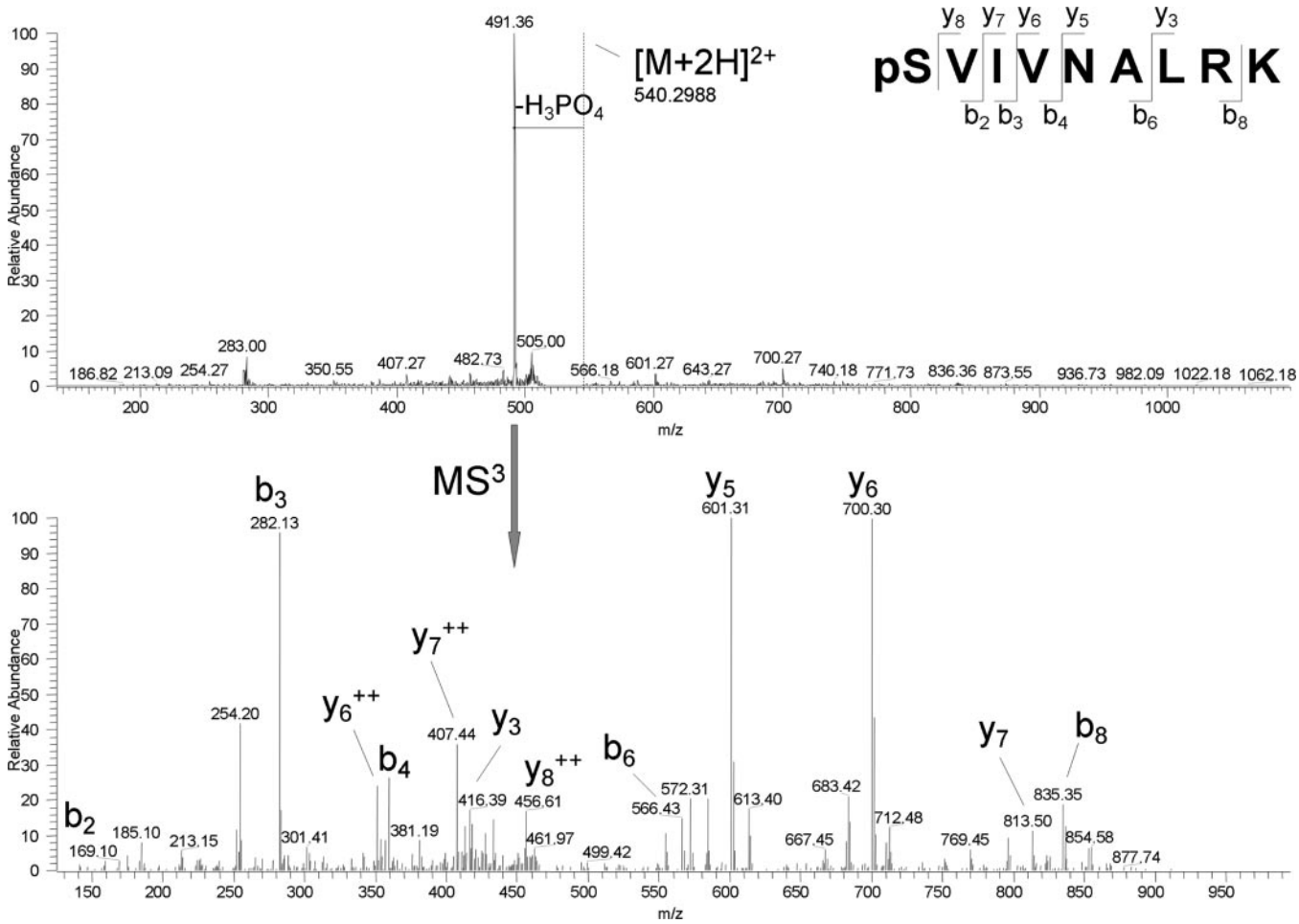

FIG. 4. **MS² spectrum of serine-phosphorylated peptide from global transcriptional regulator CodY.** Precursor ion mass was measured in the FT-ICR analyzer (mass deviation, 6.39 ppm), and the MS^2 spectrum was acquired in the LTQ mass spectrometer. *Top*, pronounced neutral loss of phosphoric acid in the MS^2 typical for Ser/Thr-phosphorylated peptides. *Bottom*, MS^3 spectrum, triggered by the neutral loss of phosphoric acid, reveals phosphopeptide identity. *pS*, phosphoserine.

amino acids, and antibiotics; protein synthesis; and the major pathways of carbohydrate metabolism in which they are significantly over-represented. The observed Ser/Thr/Tyr phosphorylations of almost the entire glycolytic pathway and several proteins of the tricarboxylic acid cycle are particularly interesting. For some glycolytic enzymes phosphorylation on serine has been shown to occur as an intermediate step in catalysis. The best studied example is the phosphoglycerate mutase from *Bacillus stearothermophilus* where it was shown that reversible conversion of 2- to 3-phosphoglycerate occurs via formation of a phosphoenzyme intermediate phosphorylated at Ser^{62} (52). Here we detected the same site occupied in the highly homologous phosphoglycerate mutase of *B. subtilis*. A similar mechanism has been proposed for the phosphoglucomutase from *Micrococcus lysodeikticus* in which the serine residue in the sequence TASHD may become phosphorylated to form the phosphoenzyme intermediate (53). We note that the phosphorylation site determined on the YbbT protein ("similar to phosphoglucomutase") in this study, SASHN, closely resembles this sequence and might therefore be involved in the catalytic activity of the enzyme. However, further functional studies will be needed to confirm this.

Catalytic activity of several related enzymes, such as 6-phosphofructo-2-kinase, has been shown to be modulated by phosphorylation in yeast (54). By extension, our data raise the possibility that a similar mechanism may be operating in *B. subtilis*. The intriguing possibility that phosphorylation of glycolytic enzymes affects their catalytic activity could shed new light on the open question of glycolytic flux regulation in Gram-positive bacteria. It is known from studies in *Lactococcus lactis* that the control coefficients of individual glycolytic enzymes on the glycolytic flux are close to zero (55). As these studies assumed a linear correlation between enzyme amount (gene expression) and enzyme activity, phosphorylation would introduce an extra level of regulation and hence refocus attention to kinases and phosphatases regulating the glycolytic flux. A number of non-glycolytic roles have been proposed recently for bacterial glycolytic enzymes (56, 57), and

an alternative possibility could be that phosphorylation directs these enzymes toward their secondary roles in the cell.

Phosphorylation of Essential Proteins—Of roughly 4100 *B. subtilis* genes, 271 were recently demonstrated or predicted to be essential in the LB medium (50). This amounts to only 6.6% of total *B. subtilis* genes, whereas 15.4% of phosphoproteins detected in this study are essential. Most of these include glycolytic enzymes, but phosphorylation in other housekeeping functions, for example protein biosynthesis, was also detected. It has been proposed that the essential bacterial genes represent the original bacterial gene set, and the non-essential genes were acquired through evolution concomitantly with the invasion of new niches (58). If this is indeed the case and if our observation of the enrichment in phosphorylation of essential genes can be shown to be generally valid, bacterial protein phosphorylation may be an ancient regulatory mechanism already present in the first primitive bacterial cells.

Phosphorylation of the Global Transcription Regulator CodY—B. subtilis protein CodY is a global transcription regulator that controls expression of over 100 genes and that has a functional counterparts in many Gram-positive bacteria (59). The CodY regulon is normally repressed during optimal growth and induced by nitrogen starvation. Thus far CodY was known to sense the nutritional state by responding to intracellular concentrations of GTP and branched-chain amino acids, which bind to the protein as allosteric regulators. We measured phosphorylation of CodY at Ser^{215}, opening another intriguing possibility for regulating this transcription regulator. Ser^{215} is situated at the extremity of α helix 9, which constitutes the DNA-binding helix-turn-helix motif (60), so its phosphorylation could present a switch for turning off the DNA binding of CodY.

New Phosphorylation Sites in the PTS Pathway—In the PTS, our dataset confirmed the known phosphorylation site of HPr (gene name *ptsH*) on Ser^{46}. It also identified two previously undetected phosphorylations: HPr at Ser^{12} and PTS enzyme I (*ptsI*) also at a serine residue. The phosphorylation of the HPr at Ser^{12} is of particular interest as this multifunctional protein has been studied in great detail previously, and yet this phosphorylation passed undetected. HPr participates in the PTS by channeling the phosphate between enzyme I and enzyme IIA, and the transferred phosphate transiently occupies the side chain of His^{15}. Phosphorylation of HPr on Ser^{46} by HPr kinase/phosphorylase has a gene-regulatory role (61) as Ser^{46}-phosphorylated HPr complexes with a transcription regulator, CcpA, that subsequently binds hundreds of regulatory *cre* sites scattered across the *B. subtilis* genome (62). Phosphorylation of HPr at Ser^{12} could affect some of the known roles of HPr, its participation in the PTS and carbon catabolite regulation, or it could have a completely new regulatory role, affected by the His^{15} phosphorylation state, as it would be difficult to imagine a simultaneous phosphorylation of Ser^{12} and His^{15} due to their close proximity.

Conclusions—In this work we present the first *in vivo* and site-specific bacterial phosphoproteome to date and demonstrate the power of analytical tools capable of analysis of global phosphorylation events in bacteria at the phosphorylation site level. These results will be useful for the wider scientific community and, we hope, will incite research on identified phosphorylation sites and function(s) of Ser/Thr/Tyr phosphorylation in *Bacillus* and other bacteria.

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