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Supplementary Table 1 Proteins identified by PMF as putative interaction partners of PrkAc.

Supplementary Fig. 1 Domain structure of PrkA. A: Schematic representation of the domains of PrkA of *L. monocytogenes*. The predicted N-terminal intracellular kinase domain is followed by a single transmembrane domain (TM). The predicted C-terminal extracellular sequence includes three PASTA domains. B: Sequence alignment of the three PASTA domains from PrkA. Each PASTA repeat consists of an α-helix and three β-strands. Predicted secondary structures are indicated below the sequences; helix denotes an α-helix and the arrows indicate β-strands. Analysis of sequences repeats were performed with RADAR (rapid automatic detection and alignment repeats) bioinformatic tool from European Bioinformatic Institute. Sequences alignment was performed with ClustalW and GeneDoc softwares. Secondary structure prediction was performed using PsiPred tool from The Bloomsbury Centre for Bioinformatics.

Supplementary Fig. 2 Spots selected for identification by PMF.

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Serine/threonine protein kinase PrkA of the human pathogen *Listeria monocytogenes*: Biochemical characterization and identification of interacting partners through proteomic approaches

Analía Lima[a], *Rosario Durán*[a], *Gustavo Schujman*[b], *María Julia Marchissio*[b], *María Magdalena Portela*[a], *Gonzalo Obal*[c], *Otto Pritsch*[c,d], *Diego de Mendoza*[b], *Carlos Cerveñansky*[a,*]

[a]*Institut Pasteur de Montevideo/Instituto de Investigaciones Biológicas Clemente Estable, Unidad de Bioquímica y Proteómica Analíticas, Uruguay*
[b]*Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET) and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina*
[c]*Institut Pasteur de Montevideo, Unidad de Biofísica de Proteínas, Uruguay*
[d]*Universidad de la República, Facultad de Medicina, Departamento de Inmunobiología, Uruguay*

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ABSTRACT

Listeria monocytogenes is the causative agent of listeriosis, a very serious food-borne human disease. The analysis of the proteins coded by the *L. monocytogenes* genome reveals the presence of two eukaryotic-type Ser/Thr-kinases (*lmo1820* and *lmo0618*) and a Ser/Thr-phosphatase (*lmo1821*). Protein phosphorylation regulates enzyme activities and protein interactions participating in physiological and pathophysiological processes in bacterial diseases. However in the case of *L. monocytogenes* there is scarce information about biochemical properties of these enzymes, as well as the physiological processes that they modulate. In the present work the catalytic domain of the protein coded by *lmo1820* was produced as a functional His_6-tagged Ser/Thr-kinase, and was denominated PrkA. PrkA was able to autophosphorylate specific Thr residues within its activation loop sequence. A similar autophosphorylation pattern was previously reported for Ser/Thr-kinases from related prokaryotes, whose role in kinase activity and substrate recruitment was demonstrated. We studied the kinase interactome using affinity chromatography and proteomic approaches. We identified 62 proteins that interact, either directly or indirectly, with the catalytic domain of PrkA, including proteins that participate in carbohydrates metabolism, cell wall metabolism and protein synthesis. Our results suggest that PrkA could be involved in the regulation of a variety of fundamental biological processes.

Abbreviations: LB, Luria Bertani; PrkAc, catalytic domain of PrkA; MBP, Myelin basic protein; STPK, serine/threonine protein kinase; STPP, serine/threonine protein phosphatase.

* *Corresponding author at:* Institut Pasteur de Montevideo, Mataojo 2020, C.P. 11400, Montevideo, Uruguay. Tel.: +598 2 5220910; fax: +598 2 5224185.
E-mail address: carlos@pasteur.edu.uy (C. Cerveñansky).

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1. Introduction

Listeria monocytogenes is a Gram positive rod-shaped bacterium that can be recovered from a wide range of sources such as soil, water, vegetation, effluents, human and animal feces and fresh and processed foods. This bacterium can tolerate hostile and stress conditions as high salt concentrations, acid pH and can grow at temperatures ranging from −1 °C to 45 °C [1]. These features allow these bacteria to survive many of the strategies used for food preservation and thus they become an important threat for human health. As a result, *L. monocytogenes* arises as an important foodborn pathogen, etiologic agent of listeriosis, ~~and~~ a sporadic but very serious disease [2]. Pregnant women, newborns, elderly and immunosuppressed individuals have predisposition to more severe presentation of the disease. In these high-risk populations, listeriosis can produce very serious clinical manifestations like septicemia, meningitis, meningoencephalitis and abortions, resulting in death in 20–30% of the cases despite early antibiotic treatment [1]. Pathogenesis of *L. monocytogenes* is mediated by its ability to effectively invade and replicate within a broad range of eukaryotic cells and to cross the intestinal barrier, blood-brain barrier, and plancental barrier in the mammalian host. *L. monocytogenes* has a relatively complex infectious cycle with different stages: internalization in host cells, intracellular proliferation and intercellular spread. Each stage of the intracellular parasitism is dependent upon the differential expression of distinct virulence factors [3].

The extraordinary capacity of *L. monocytogenes* to adapt and respond to environmental changes seems to be related to an extensive repertoire of predicted regulatory proteins, including different RNA polymerase sigma factors, transcription factors and protein phosphorylation systems [4]. Protein phosphorylation is a major mechanism in signal transduction processes by which environmental stimuli are translated into cellular responses and represents one of the most important post-translational modifications regulating enzyme activities and protein interactions [5,6]. Signal transduction in prokaryotes is predominantly accomplished by the so called two-component systems, consisting of His-kinase sensors and their associated response regulators [7]. In contrast, in eukaryotes such signaling pathways are mainly carried out by Ser/Thr or Tyr-kinases [8]. Long time thought to be exclusive to eukaryotes, a bulk of evidence raised from genome sequence data now indicates that Ser, Thr, and Tyr phosphorylation is also widespread in prokaryotes [9]. These eukaryotic-like signaling systems have been shown to control essential processes in bacteria, including development, cell growth, stress responses, central and secondary metabolism, biofilm formation, antibiotic resistance, and virulence [9–15]. In the case of *L. monocytogenes* the presence of eukaryotic-like phosphorylation systems has been predicted by genome analysis. In particular, it was reported that the *stp* gene (*lmo1821*) encodes a functional Ser/Thr protein phosphatase (STPP) required for growth of *L. monocytogenes* and virulence in murine model of infection. In addition, the elongation factor EF-Tu was described as a target for this phosphatase [16]. However, there is no information regarding the corresponding phosphorylating enzymes, endogenous substrates and their role in bacteria physiology and physiopathology.

In the present work we report the cloning, expression and purification of the catalytic domain of the gene product of *lmo1820*, named PrkA, a putative transmembrane Ser/Thr protein kinase (STPK) coded by the *L. monocytogenes* genome. We produced the catalytic domain of PrkA (PrkAc) as a functional enzyme able to phosphorylate an exogenous substrate at Ser and/or Thr residues. We also demonstrate that PrkAc is autophophorylated at specific conserved Thr residues. Finally, as a first attempt in deciphering the potential role of PrkA, we identified 62 proteins that possibly interact, directly or indirectly, with the phosphorylated catalytic domain. These putative interaction partners participate in a wide range of cellular processes, indicating that PrkA could have a role in the regulation of a diversity of essential biological functions in *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains, vectors, and culture conditions

Escherichia coli DH5α and *E. coli* M15[pREP] (Qiagen) were used for plasmid maintenance and protein expression, respectively. The plasmid pQE32 (Qiagen) was used as protein expression vector. *E. coli* strains were cultured on Luria-Bertani (LB) agar or broth. When required, media were supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin. *L. monocytogenes* EGDe was cultured on LB agar or broth supplemented with 50 mM glucose.

2.2. General genetic techniques

Genomic DNA from *L. monocytogenes* EGDe was prepared by heating bacterial colonies in ultrapure water at 100 °C for 5 min. Cellular debris were discarded by centrifugation a 10,000 *g* and the supernatant, containing genomic DNA, was used as template for PCR reactions. Plasmid DNA from *E. coli* cells was prepared with Wizard Plus Minipreps DNA purification system (Promega). DNA fragments from agarose gels were obtained using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). DNA digestion with restriction enzymes, ligation reactions with T4 DNA ligase and agarose gel electrophoresis were carried out according to methods described by Sambrook et al. [17]. Transformation of *E. coli* competent cells with plasmid DNA was performed using the $CaCl_2$ method [17].

2.3. Sequence analysis

Protein sequence of the potential STPK PrkA (*lmo1820*) from *L. monocytogenes* EGDe was obtained from Listilist web site (http://genolist.pasteur.fr/ListiList/). Multiple sequence alignment of PrkA with other characterized STPKs from related microorganism was carried out using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw/). Analyses related to sequence conservation were performed using the Genedoc software http://www.nrbsc.org/gfx/genedoc/. Other bioinformatics tools (TMHMM server v 2.0, RADAR available at http://www.expasy.ch/tools/) were used for the prediction of transmembrane domains and sequence repeats.

2.4. Cloning, expression and purification of PrkAc

PrkAc (amino acids 1–338) was produced as a His_6-tagged protein in *E. coli*. For that purpose, DNA fragment corresponding

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to PrkAc was synthesized using genomic DNA from *L. monocytogenes* EGDe as a template and the following primers: 1820CU, 5′-GATGCTGGATCCTGATTGGTAAGCGATT-3′ and 1820CL, 5′-AACAATGTCGACCTATTTCTTTTTCTTGCTCAT-3′. Primers 1820CU and 1820CL contained the *Bam*HI and *Sal*I restrictions sites, respectively. After digestions with the corresponding restriction enzymes, the PCR product was cloned into pQE32 vector (Qiagen). The resulting plasmid was introduced into *E. coli* M15[pREP4] for protein expression. The sequence of the cloned protein was verified by DNA sequencing.

The expression strain was grown at 37 °C until mid-log phase in LB broth supplemented with ampicillin and kanamycin. Induction of protein expression was conducted for 4 h at 37 °C after the addition of 1 mM isopropyl-β-thiogalactopyranoside. Then, bacterial pellets were resuspended in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol and lysed by sonication on ice followed by centrifugation. The His_6-tagged proteins were purified under native condition by Ni^{2+}-affinity chromatography according to the manufacturer instruction (Qiagen) followed by dialysis against 50 mM HEPES, pH 7.2. Protein purification was monitored by SDS-PAGE [18] and protein concentrations were determined by Bradford assays [19].

2.5. *In vitro phosphorylation and de-phosphorylation assays*

Protein kinase assay was carried out using recombinant PrkAc in 50 mM HEPES buffer, pH 7.0, containing 1 mM DTT, 2.5 mM $MnCl_2$, and 100 μM ATP. Myelin basic protein (MBP) was used as substrate at a concentration of 25 μM (kinase-substrates molar ratios of 1:10). Reactions were performed at 37 °C for 30 min. Phosphorylation of MBP at peptide 30–41 was monitored by MS measurements after tryptic digestion.

For autophosphorylation assay, PrkAc was pre-treated with alkaline phosphatase from calf intestine (Roche Diagnostic) and its de-phosphorylation state was confirmed by MS of digested protein. De-phosphorylated kinase was isolated from the mixture using Ni^{2+}-affinity resin and incubated at 37 °C in presence of $MnCl_2$, ATP as described above. Autophosphorylated peptides were detected by MS after tryptic digestion.

2.6. *Sample preparation for MS analysis*

Proteolytic digestion was carried out by incubating the proteins with trypsin (sequence grade, Promega) in 50 mM ammonium bicarbonate, pH 8.3, for 2 h at 37 °C (enzyme–substrate ratios 1:10). The β-elimination reactions at phosphoresidues were performed by treating 2 μg of PrkAc tryptic peptides with a saturated solution of $Ba(OH)_2$ at room temperature for 4 h as previously reported [20]. Then, the samples were acidified with 10% TFA.

For analysis of proteins obtained from acrylamide gels, selected spots or bands were manually cut and in-gel digested with trypsin (sequence grade, Promega) as described [21]. Peptides were extracted from gels using aqueous 60% ACN containing 0.1% TFA and concentrated by vacuum drying.

Prior to MS analyses, samples were desalted using C18 reverse phase micro-columns (Omix®Tips, Varian) and eluted directly onto the sample plate for MALDI-MS with CHCA matrix solution in aqueous 60% ACN containing 0.1% TFA.

2.7. *MALDI-TOF MS analysis*

Mass spectra of peptides mixtures were acquired in a 4800 MALDI TOF/TOF instrument (Applied Biosystems) in positive ion reflector mode. Mass spectra were externally calibrated using a mixture of peptide standards (Applied Biosystems). MS/MS analyses of selected peptides were performed.

Proteins were identified by the database searching of measured peptide *m/z* values using the MASCOT program (Matrix Science http://www.matrixscience.com/search form select.html), and based on the following search parameters: monoisotopic mass tolerance, 0.05 Da; fragment mass tolerance, 0.3 Da; partial methionine oxidation, cysteine carbamidomethylation and one missed tryptic cleavage allowed. Protein mass and taxonomy were unrestricted. Significant scores ($p<0.05$) were used as criteria for positive protein identification.

Phosphorylation state of presumptive phosphopeptides was confirmed by MS/MS experiments. The identification of phosphorylated residues was achieved by MS/MS analysis of peptides treated with $Ba(OH)_2$.

2.8. *Preparation of L. monocytogenes protein extracts*

L. monocytogenes were grown in LB supplemented with 50 mM glucose at 37 °C until mid-log phase. Pellets were resuspended in 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1% glycerol, 10 μg/ml proteases inhibitor mix (GE Healthcare). Bacterial suspension was treated with 1 mg/ml lysozyme and incubated on ice for 30 min. Then, cells were disrupted by sonication on ice. After treatment with 10 μg/ml RNAse and 5 μg/ml DNAse, cells debris was removed by centrifugation at 10,000 *g* for 30 min at 4 °C and the supernatants were collected and stored at −80 °C. Total protein concentration was determined using 2D-Quant kit (GE Healthcare).

2.9. *Surface plasmon resonance analysis*

Surface plasmon resonance experiments were performed on a BIAcore 3000 instrument (BIAcore, Piscataway, NJ). PrkAc was immobilized using standard amine-coupling procedures (Amine Coupling Kit, BIAcore) on a CM5 sensorchip at pH 4 to a final density of 8800 resonance units (RU). Then, the instrument was primed with running buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.005% Tween 20). A flow cell activated and blocked with ethanolamine was left as a control surface for non-specific binding.

Forty microlitres of 15 μg/ml of a *L. monocytogenes* total protein extract were injected onto the surfaces. Binding experiments were performed at 25 °C at a flow rate of 10 μl/min during 240 s. After extensive washing with running buffer, ligands were eluted using 50 μl of 20 mM glycine pH 3 or 1 M NaCl at flow rate of 100 μl/min during 30 s in two independent experiments. All data processing was carried out using the BIAevaluation 4.1 software provided by BIAcore. Binding responses were first double-referenced by subtracting

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signals corresponding to both reference flow cell and from the average of blank (buffer) injections.

2.10. *Preparation of immobilized PrkAc affinity resin*

Recombinant PrkAc was covalently coupled to HiTrap NHS-activated HP (Amersham Biosciences) following the instructions provided by the manufacturer. Briefly, the resin was washed with cold 1 mM HCl and activated with coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Then, 400 μg of PrkAc was added to the activated resin and incubated for 4 h at 4 °C with gentle agitation. Washing and blocking of the resin unreacted groups was performed by alternated washes with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 and 0.1 M CH_3COONa, 0.5 M NaCl, pH 4. The same process was carried out to prepare a control resin, but omitting the addition of PrkAc in the coupling step.

Covalent binding of PrkAc to resins was confirmed by proteolytic digestion with trypsin and MS analysis. The activity of the covalently bound PrkAc was also tested using MBP as substrate and monitoring its phosphorylation by MS analysis.

2.11. *Affinity chromatography*

L. monocytogenes protein extract (600 μl, 7 mg/ml) prepared as described was added to immobilized PrkAc and control resin (previously equilibrated with 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% glycerol) and incubated for 4 h at 4 °C with gentle agitation. Then, resins were extensively washed with 10 mM HEPES, 150 mM NaCl, pH 8.3 and finally bound proteins were eluted with 20 mM glycine pH 3.0. The chromatographic fractions were analyzed by 12.5% SDS-PAGE followed by silver staining. Additionally, eluted fractions were concentrated and analyzed by 2D electrophoresis. Two affinity chromatography experiments were run independently with different cell extracts.

2.12. *2D electrophoresis*

First dimension was performed with commercially available IPG-strips (7 cm, linear 3–10, GE Healthcare). Eluted protein fractions were purified and concentrated with 2-D Clean-Up kit (GE Healthcare) and dissolved in 125 μl of rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer 3–10 [GE Healthcare], 0.002% bromophenol blue). Samples in rehydration solution were loaded onto IPG-strips by passive rehydration during 12 h at room temperature.

The isoelectric focusing was done in an IPGphor Unit (Pharmacia Biotech) employing the following voltage profile: constant phase of 300 V for 30 min; linear increase to 1000 V in 30 min; linear increase to 5000 V in 80 min and a final constant phase of 5000 V to reach total of 6.5 kVh. Prior running the second dimension, IPG-strips were reduced for 15 min in equilibration buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) supplemented with DTT (10 mg/ml) and subsequently alkylated for 15 min in equilibration buffer supplemented with iodoacetamide (25 mg/ml). The second-dimensional separation was performed in 12.5% SDS-PAGE using a SE 260 mini-vertical gel electrophoresis unit (GE Healthcare). The size markers used were Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare).

The gels were silver stained according to protocols described [22]. Images were digitalized using a UMAX Power-Look 1120 scanner and LabScan 5.0 software (GE Healthcare).

3. Results and discussion

3.1. *Sequence analysis*

The analysis of the *L. monocytogenes* EGDe genome revealed the presence of two putative STPKs (*lmo0618* and *lmo1820*) and one STPP (*lmo1821*). In the 10.2 kbp region that encloses the gene coding PrkA (*lmo1820*) eight open reading frames are found (http://genolist.pasteur.fr/ListiList/) (Fig. 1). This gene cluster also includes the gene *lmo1821* and other genes involved in information pathways (DNA, RNA and protein metabolism and modification) (*lmo1819*, *lmo1822*, *fmt*, and *priA*) and intermediary metabolism (*lmo1818* and *lmo1825*). The presence in the same genome region of a STPP gene preceding the STPK gene was also found in other bacteria suggesting a functional association between theses enzymes [23–27]. Particularly it has been observed that such STPK/STPP couples act as functional pairs in *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Bacillus subtilis* [23,25,28,29].

The STPK PrkA is a predicted 655 amino acids transmembrane protein, with a theoretical molecular mass of 72 kDa and a pI value of 4.99. Sequence analysis showed the presence of a pattern of basic residues followed by a predicted transmembrane domain suggesting that the N-terminal region (residues 1–338) is orientated toward the cytoplasm [30]. It was also observed that PrkA N-terminal sequence contains a predicted STPK that exhibits all the conserved subdomanis (subdomains I to V, VIa, VIb and VII to XI) and the nearly invariant residues that define the Hanks family of eukaryotic protein kinases [8] (Fig. 2). Protein sequence alignments showed that the putative kinase domain of PrkA has high homology with the catalytic domain of other well studied bacterial STPK, such as PrkC from *B. subtilis* (68% identity), StkP form *Streptococcus pneumoniae* (53% identity), Stk1 from *S. aureus* (49% identity) and PknB from *M. tuberculosis* (46% identity) (Fig. 2).

Analysis of the C-terminal domain sequence of PrkA showed the presence of several copies of PASTA domains (Penicillin-binding protein and Ser/Thr kinase Associate) (supplementary Fig. 1). This domain interacts with peptidoglycan fragments and β-lactamic antibiotics and is present in high molecular weight penicillin-binding proteins and eukaryotic-like STPKs of a variety of pathogens [31,32]. This structural organization, with extracellular PASTA domains and intracellular kinase domain is also well conserved in different prokaryotic STPKs, including PknB from *M. tuberculosis*, *Corynebacterium glutamicum* and *S. aureus*, PrkC from *B. subtilis* and StkP from *S. penumoniae* [23,24,33–35], pointing to the regulation of related processes by protein phosphorylation in response to similar stimuli in these microorganisms. STPKs from this group participate in the regulation of diverse bacterial processes including growth, cell division, developmental states, central and secondary metabolism and expression of virulence factors [13–15].

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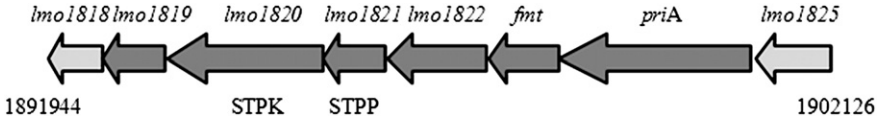

Q1 **Fig. 1 – Organization of the genome region enclosing the gene that encodes for the putative Ser/Thr protein kinase PrkA. Arrows indicate the orientation of transcription. This region encodes six ORFs involved in information pathways (dark gray) and two ORFs involved in secondary metabolism (light gray). *lmo1818*: similar to ribulose-5-phosphate 3-epimerase; *lmo1819*: similar to ribosome associated GTPase; *lmo1820*: PrkA, similar to putative Ser/Thr-specific protein kinase; *lmo1821*: similar to putative phosphoprotein phosphatase; *lmo1822*: similar to RNA-binding Sun protein; *fmt*: similar to methionyl-tRNA formyltransferase; *priA*: similar to primosomal replication factor Y; *lmo1825*: similar to pantothenate metabolism flavoprotein homolog; STPK: Ser/Thr protein kinase; STPP pSer/pThr protein phosphatase.**

3.2. *PrkAc expression and purification*

In order to perform the characterization of the STPK PrkA, we expressed the entire N-terminal region encompassing the kinase domain as a His_6-tagged protein (PrkAc). DNA sequence corresponding to amino acids 1–338 was amplified by PCR and partial sequencing assured error-free amplification and in-frame fusion with the His_6-tag of the expression vector.

Purification of PrkAc was performed under native conditions using Ni^{2+}-NTA affinity resin. SDS-PAGE analysis showed a band that migrates according to the predicted molecular mass of the recombinant protein (39 kDa for the catalytic domain) and two additional bands ranging from 41 to 43 kDa (Fig. 3). All these proteins were identified by PMF as PrkA demonstrating that the protein expressed in *E. coli* has at least three isoforms with different migration behavior in SDS-PAGE.

The recombinant protein PrkAc was examined for its ability to phosphorylate the exogenous substrate MBP. Comparison of mass spectra of digested MBP after and before incubation with PrkAc in the presence of ATP and Mn^2 revealed that sequence 30–41 is phosphorylated by the kinase. Signal of native sequence ($m/z = 1339.61$) present in control spectra decreased after phosphorylation reaction and concomitantly a signal with a mass increment of 80 Da ($m/z = 1419.68$) became apparent (Fig. 4). This particular MBP peptide was found to be systematically and extensively phosphorylated by several mycobacterial STPKs. Its detection by MS was previously reported as a sensitive marker of kinase activity [36]. Phosphorylation of MBP tryptic peptide 30–41 by PrkAc was further confirmed by MS/MS analysis (Fig. 4). The presence of daughter ions with mass differences of 80 Da (loss of HPO_3) and 98 Da (loss of H_3PO_4) is characteristic of phosphorylated peptides [36,37]. These results clearly demonstrate that PrkAc was produced in *E. coli* as a functional STPK able to phosphorylate the exogenous substrate MBP. The fact that PrkAc phosphorylates the same MBP peptide than mycobacterial protein kinases probably reflects some specificity of bacterial kinases towards this sequence.

3.3. *Identification of phosphorylated peptides and residues in PrkAc*

The overall phosphorylation status of the recombinant kinase was tested by MALDI-TOF mass measurements of tryptic digestions of PrkAc before and after the treatment with alkaline phosphatase. Results obtained from spectra comparison allowed us to predict the presence of phospho-Ser and phospho-Thr containing peptides ($m/z = 3733.72$, $m/z = 3813.96$, and $m/z = 3893.90$ could be assigned to the mono-, di-, and tri-phosphorylated tryptic peptide 160–183 respectively) (Fig. 5). Additionally, the multiple phosphorylated state of these peptides was confirmed by MS/MS analyses (data not shown). It is interesting to note that this multiple phosphorylated peptide is enclosed within the conserved motifs DFG and PE of Hanks kinases corresponding to the activation loop in several STPK from related bacteria [8,23,25,36,38–40].

The identification of phosphorylation sites by MS/MS analyses is usually challenging because fragmentation of phosphopeptides is mainly dominated by the neutral loss of phosphate group. This fact precludes the detection of sequence-specific ion signals rendering difficult the localization of modification sites [36]. For that reason, we treated the phosphorylated peptides with $Ba(OH)_2$ to generate de-hydro amino acids from phospho-Ser and phospho-Thr residues by β-elimination of H_3PO_4. Such derivatives have better properties for MS/MS experiments. Moreover they show a mass difference of 18 Da compared to the parent amino acid residue, thus becoming a useful tag for phosphoresidue identification [41]. The spectrum of $Ba(OH)_2$ treated peptides showed signals 18, 36 and 54 Da lower than the expected for native peptides 160–183, indicating the presence of species that have been generated by multiple β-elimination of phosphate group (Fig. 6).

The phosphorylation sites were assigned by manual inspection of MS/MS spectrum of the ion generated after β-elimination reaction of the tri-phosphorylated peptide. This spectrum shows mostly y-ions and the presence of signals with mass differences of 18 Da (and multiple thereof) in relation to the theoretical expected values, was clearly detected allowing the unequivocally identification of modified residues (Fig. 6). The results allowed us to identify the phosphorylation sites as Thr171, Thr174 and Thr176 within the sequence 160–183 of PrkA activation loop. At least two of this Thr residues are highly conserved in the activation loop sequence of other bacterial STPKs and its phosphorylated state has been reported [23,35,36,38,40]. In addition, it was demonstrated for some STPKs, such as PrkC from *B. subtilis* and PknB from *M. tuberculosis*, that the phosphorylation of these conserved Thr residues in the activation loop regulates kinase activity [23,35].

To test if phosphorylation of the activation loop sequence was a result of an autocatalytic reaction, the recombinant kinase was de-phosphorylated using alkaline phosphatase, purified using Ni^{2+}-NTA resin and re-incubated in the presence of ATP and Mn^{2+}. The phosphorylation status of PrkAc was followed by

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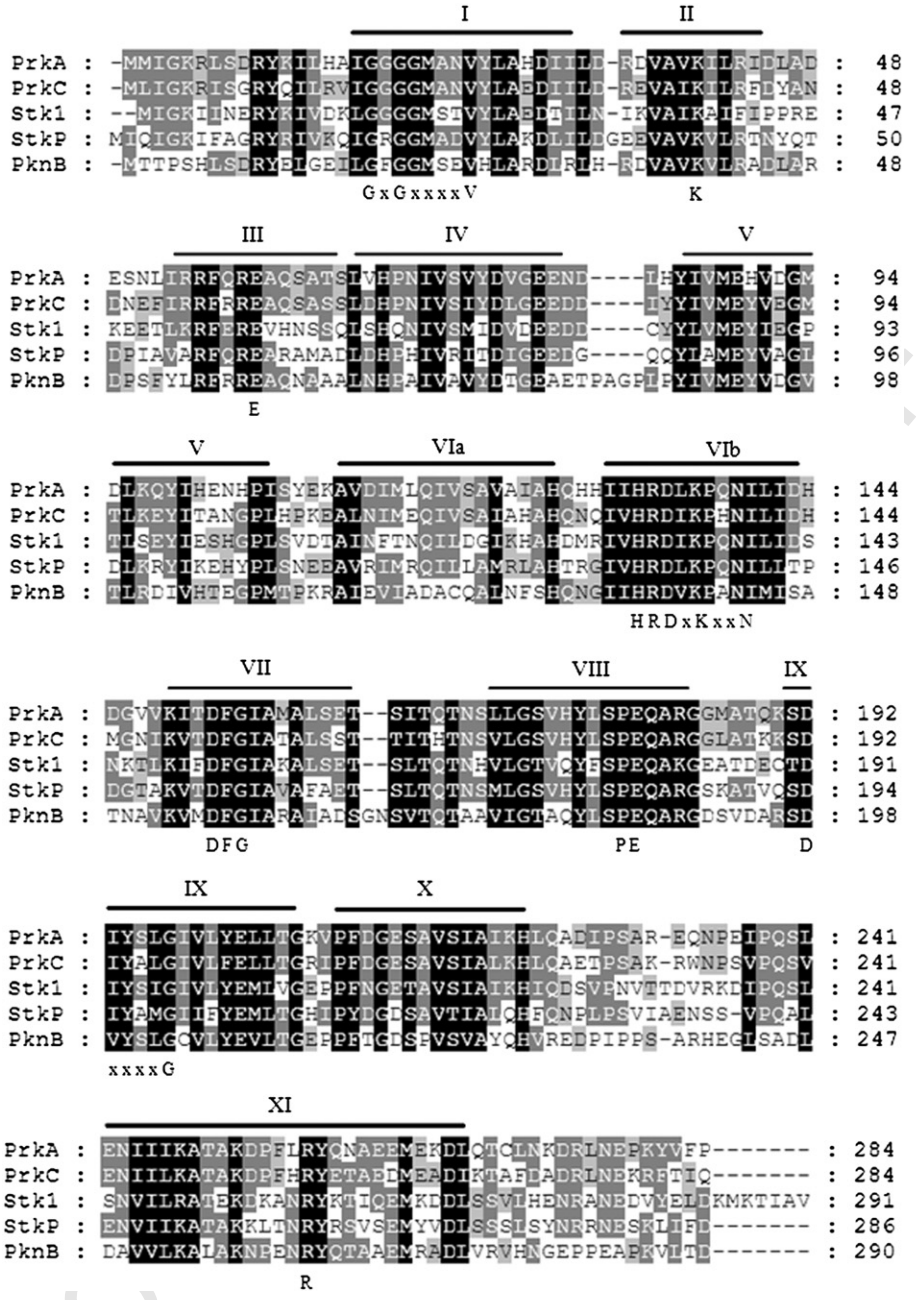

Fig. 2 – Protein sequence alignment of the N-terminal domain of PrkA and catalytic domains of other characterized bacterial Ser/Thr protein kinases. PrkA, putative STPK from *L. monocytogenes*; PrkC, from *B. subtilis*; Stk, from *S. aureus*; StkP, from *S. pneumoniae*; and PknB, from *M. tuberculosis*. Sequences alignment was performed with ClustalW and GeneDoc softwares. Sequences showing 100% of conservation are shaded in black (identical residues and conservative changes). Sequences showing more than 60% and 40% of conservation are indicated in dark and light gray respectively. Sub-domains I-IX that define the Hanks family of eukaryotic-like protein kinases are indicated above and nearly invariant residues are indicated below the alignment.

MS analysis after proteolytic treatment. Spectra analysis showed that phosphatase treatment results in activation loop de-phosphorylation, indicated by the disappearance of phosphorylated species and the increase of native peptide *m/z* signal. After incubation of the de-phosphorylated enzyme with ATP the activation loop phosphopeptides were clearly detected in the mass spectrum, indicating that PrkAc presented autocatalytic activity (data not shown).

The activation loop phosphorylation status is important to control the active/inactive conformational switch in numerous kinases. A wide range of regulatory mechanism has been suggested for this loop, such as the contribution to the appropriate alignment of the catalytic residues and the correction of the relative orientation of different domains allowing the binding of the protein substrate and/or ATP [42]. The relevance of the activation loop phosphorylation has been

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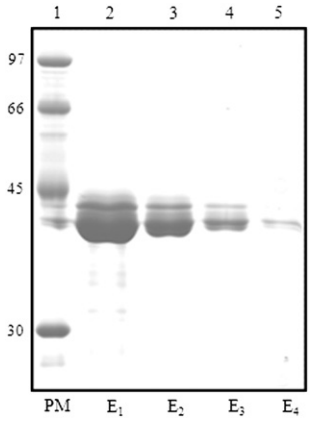

Fig. 3 – Over-expression and purification of His_6-tagged PrkAc. Proteins were purified with Ni^{2+}-NTA resin, separated on 12.5% SDS-PAGE and stained with Coomassie blue. Lane 1: molecular weight marker (Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis); lanes 2–5: different fractions eluted with 500 mM imizadol. At least 3 bands ranging from 39 to 43 kDa were detected in the eluted fractions and were identified as PrkA from *L. monocytogenes* by PMF.

demonstrated by using point mutation in PknB from *M. tuberculosis* and PrkC from *B. subtilis* [23,35]. In addition our group has demonstrated that phosphorylated residues in the activation loop are not only important for enzyme activity but also defines a high affinity docking site that is relevant for substrate recruitment [43]. Considering these evidences from homologous proteins, we can suggest that the very well conserved phosphorylation pattern here reported for PrkA, participates in activity control and perhaps also in substrate recruitment by protein interactions mediated by specific phospho-residues recognition.

3.4. *Identification of putative interacting partners of PrkAc*

As a first approach to reveal possible interactions between phosphorylated PrkAc and proteins from *L. monocytogenes* cellular extracts, we used a surface plasmon resonance strategy. These experiments allowed us to determine that immobilized PrkAc interacted with components of *L. monocytogenes* protein extract (data not shown).

In order to identify the proteins that possibly interact with PrkAc we carried out affinity chromatography experiments using the conditions obtained from surface plasmon resonance experiments. For that purposes, we first immobilized recombinant PrkAc to a Hi-trap NHS-activated resin HP (Amersham Bioscience). A fraction of the resin submitted to the process of immobilization was digested with trypsin and analyzed by MS to confirm the coupling of PrkAc. Only tryptic masses from PrkAc were detected, discarding the presence of significant amounts of contaminating proteins. The incubation of the covalently bound kinase with MBP under phosphorylation conditions showed that the immobilized protein was an active enzyme (data not shown).

To recover either individual proteins or protein complexes that bind to PrkAc, we incubated the modified and control

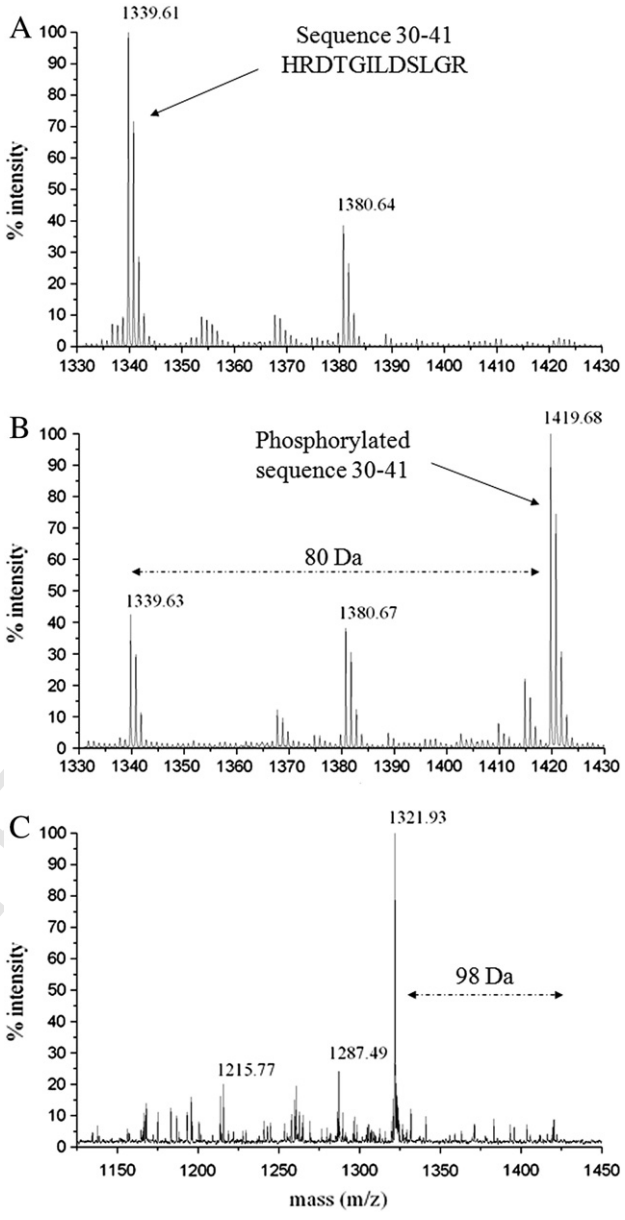

Fig. 4 – Activity of PrkAc using myelin-basic protein (MBP) as a substrate. Mass spectra of MBP digest before (A) and after (B) incubation with the kinase in the presence of ATP and Mn^{2+}. Arrows indicate the tryptic peptides 30–41 from native MBP and the presumptive mono-phosphorylated species. The MS/MS analysis of *m/z* = 1419.68 shows the neutral loss of 98 Da characteristic of phosphopeptides (C).

resin with a soluble protein extract from *L. monocytogenes* EGDe. After extensive washing the ligands were eluted using acid pH. The different fractions of the affinity chromatography were primarily analyzed by one-dimensional SDS-PAGE and visualized by silver staining. From these analyses we could observed that many proteins were retained by PrkAc resin while we did not detect proteins in control resins (data not shown).

In order to achieve a better resolution, eluted protein were separated by 2D electrophoresis. Analysis of 2D gels allowed us to detect a specific protein profile of eluted proteins in

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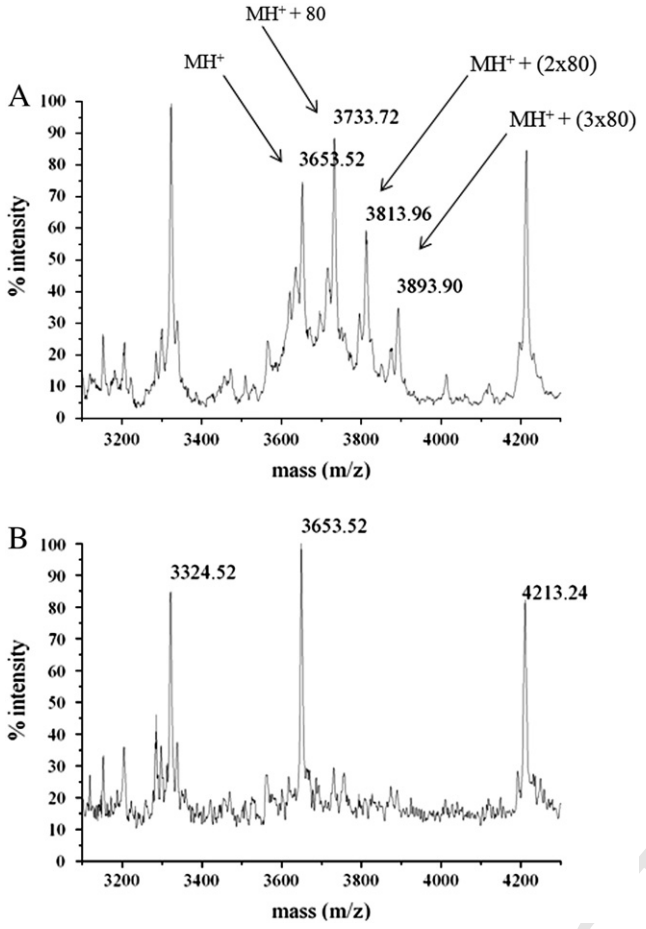

Fig. 5 – Detection of phosporylated peptides in PrkAc. Mass spectra of tryptic digestion of PrkAc before (A) and after (B) the treatment with alkaline phosphatase. Mass signals corresponding to native peptide 150–183 (MH^+) and its mono-, di- and tri-phoshorylated ions, showing a mass shift in 80 Da and multiples thereof, are indicated with arrows. The multiple phosphorylation of the sequences 150–183 was confirmed by the disappearance of the corresponding ions from the spectrum after phosphatase treatment.

independent experiments that clearly differed from the 2D profile of total cellular extracts (data not shown). Spots detected in all replicates were processed for protein identification by PMF (Fig. 7 and supplementary Fig. 2). This strategy allowed the identification of 62 proteins that possibly interact, directly or indirectly, with PrkAc. For each protein identified, supplementary Table 1 reports protein Mascot scores and ion scores generated from fragmentation of selected *m*/z values, protein sequence coverage, and other parameters used in the identification. Table 1 displays the complete list of PrkAc putative interactors identified in this study, grouped according to their functional category. The two largest groups were composed of proteins functionally related to the metabolism of carbohydrates (26%) and protein synthesis (19%) (Fig. 8). This is followed by proteins involved in transport and binding of proteins and lipoproteins (10%) and in cell wall metabolism (9%). A primary conclusion that arises from the diversity of proteins identified as potential interaction partners of PrkAc could be that the signal transduction pathways mediated by this STPK in *L. monocytogenes* could be affecting a great variety of fundamental biological functions.

Since the immobilized protein is the autophosphorylated catalytic domain of a STPK, we consider the possibility that some of the potential interacting partners were also substrates of the kinase. Therefore we searched reported phosphoproteomes to see if the identified proteins were phosphorylated at Ser or Thr in other microorganism. We found that 48% of the proteins were described to be phosphorylated in at least one of the following microorganisms: *C. glutamicum*, *B. subtilis*, *E. coli*, *M. tuberculosis*, *Pseudomonas aeruginosa*, *P. putida*, *Lactococcus lactis*, *S. pneumoniae*, and *Campylobacter jejuni* [44–53].

It is also important to note that many of these putative partners were reported as the proteins most frequently identified in differential expression proteomic analysis based on 2D gel approaches [54,55]. If the identification of these proteins represents a technical artifact or reveals that they participate in a general cell mechanism is still a matter of debate [54,55]. Even when our experimental approach points to a specific interaction of these proteins with PrkAc, we have to be very careful with the interpretation of these results. In addition to these frequently detected proteins, less abundant regulatory proteins were also identified as possible interactors of PrkAc.

The list of proteins and protein families identified provides information regarding possible functions of PrkAc. In the following paragraphs we focus on some of the potential interaction partners of PrkAc that are related to STPKs function in other organisms and whose relevance has been reported or strongly suggested.

3.4.1. *Proteins involved in the carbohydrate metabolism*

We identified 15 proteins related to the glycolytic pathway and the tricarboxylic acid (TCA) cycle. Some of them (aldolase, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvate kinase, lactate dehydrogenase, acetate kinase, dihydrolipoamide dehydrogenase and α-cetoglutarate dehydrogenase) were found to be phosphorylated at Ser, Thr or Tyr residues trough phosphoprotemic studies in other microorganisms [44–53]. It was also proved that the transcriptional profile of two enzymes involved in the TCA cycle (dihydrolipoamide succinyltransferase and oxoglutarate dehydrogenase E1) is affected by the STPK PknB from *S. aureus* [56]. Additionally, in *M. tuberculosis* and *C. glutamicum* it has been demonstrated that the regulation of TCA cycle is mediated by STPKs [57,58]. In these bacteria, the STPKs PknB and PknG phosphorylate a protein containing a FHA domain (GarA y OdhI in *M. tuberculosis* and *C. glutamicum* respectively) which in their de-phosphorylated forms inhibit the enzyme 2-oxoglutarato dehydrogenase [57,58]. FHA domains are small protein modules that mediate protein–protein interactions in the STPK-mediated signal transduction pathways through the recognition of specific phosphorylated residues [59]. Genome sequence analyses have revealed that all members of the order actinomycetales present GarA-homologous proteins which show strong sequence conservation at the C-terminus FHA domain [43]. However, the analysis of the proteins coded by the *L. monocytogenes* genome does not predict the presence FHA-containing proteins. Therefore, the STPK PrkA in *L. monocytogenes* could be involved in the modulation of the TCA cycle through a

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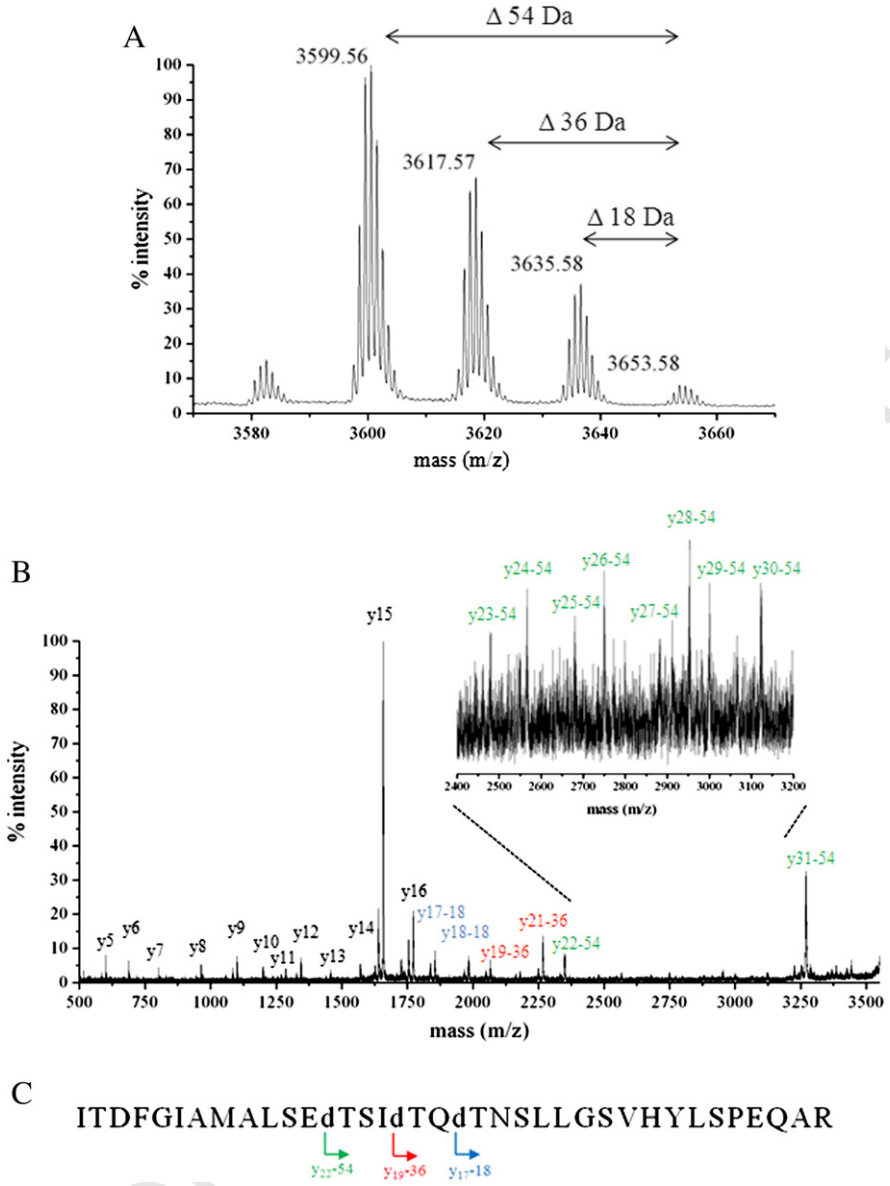

Fig. 6 – Identification of phosphorylation sites by MS/MS analysis. (A) Spectrum of tryptic digestion of PrkAc after treatment with $Ba(OH)_2$. The appearance of mass signals differing in 18 Da, 36 Da and 54 Da from native peptide 160–183 confirmed the β-elimination of one, two and three phosphate groups respectively. (B) MS/MS analysis of peptide generated from tri-phosphorylated species after β-elimination reaction. The occurrence of y-ions with mass difference of 18 Da (and multiples) allowed the identification of de-hydrated Ser and Thr residues generated from previously phosphorylated residues by β-elimination reaction. (C) 160–183 sequence showing the identified modified residues.

different mechanism from that described in the members of the order actinomycetales.

3.4.2. *Proteins involved in cellular information pathways (DNA, RNA and protein synthesis and related proteins)*

We identified the following proteins that are implicated in DNA and RNA synthesis: DNA polymerase, RNA polymerase (α and β subunits), transcriptional repressor Rex and the RNA binding protein Sun. The RNA polymerase was found phosphorylated by phosphoproteomic approaches in *M. tuberculosis* and *S. pneumoniae* [49,52].

One of the most interesting proteins arising from this study is the RNA binding protein Sun. The gene that codes for Sun (*lmo1822*) is located in the same genomic region and adjacent to the genes *lmo1820* and *lmo1821* (coding for PrkA and Stp respectively), probably organized in an operon. This observation suggests that both proteins could be genetically and functionally linked. The fact that both STPK and its substrates are encoded in the same genomic region is recurrent for many STPKs from many organisms [60–63].

We also detected 9 proteins involved in the biosynthesis of proteins, as ribosomal proteins, aminoacyl t-RNA synthetases, the translation initiation factor InfB, and the translation elongation factors EF-Tu and EF-G. The translation initiation and elongation factors and the isoleucyl-tRNA synthetase were found to be phosphorylated in other bacteria [44–46,48–53].

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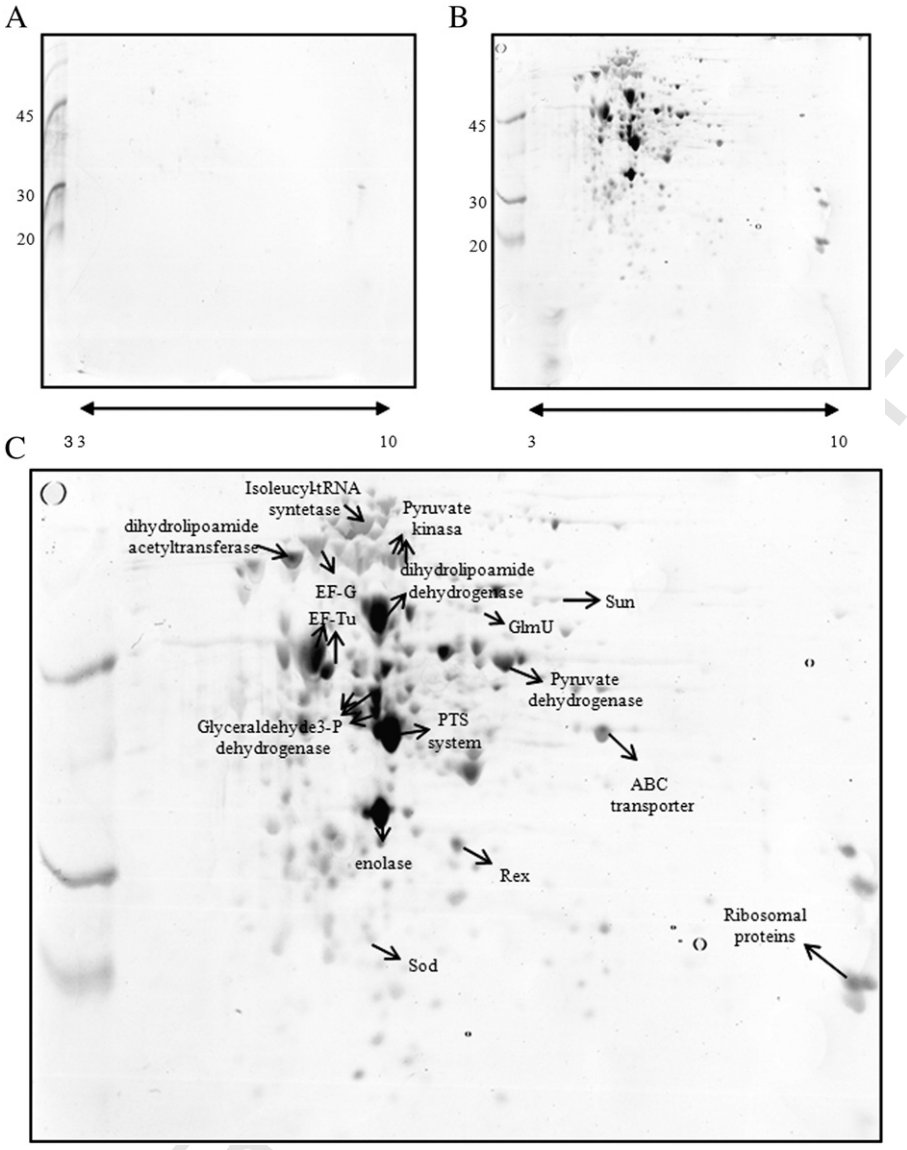

Fig. 7 – 2D electrophoresis of eluted proteins from a PrkAc affinity chromatography. Representative gels showing proteins eluted from a control resin (A) and from a resin with PrkAc immobilized (B). Some of the proteins identified by PMF are indicated (C).

Additionally, the elongation factors EF-Tu and EF-G were described as substrates of the STPK and the STPP from *B. subtilis* [60,64], and EF-Tu was also recognized as the substrate of the STPP from *L. monocytogenes* [16]. Taking into account that EF-Tu is indeed phosphorylated in *L. monocytogenes* that only encodes two STPKs, the identification of this protein in PrkA interactome suggest that it might be an endogenous substrate of this kinase.

3.4.3. *Proteins involved in the cell wall metabolism*

In this study we identified 5 proteins that participate in the cell wall metabolism: the cell shape determining proteins MreB and Mbl, and the proteins involved in the peptidoglycan synthesis, ~~N-acetylglucosaminyl transferase~~, UDP-N-acetylglucosamine pyrophosphorylase (GlmU) and glucose-1-phosphate thymidylyltransferase. Several STPKs, in particular the ones that have PASTA domains as sensor extracellular domains, have been implicated in the regulation of the cell wall metabolism. Different proteins related to the growth and cellular division were identified as substrates of STPKs, as DivA, PbpA ,FtsZ and GlmU from *M. tuberculosis* and GlmS from *S. pneumoniae* [61,65–69]. GlmU was also found as a phosphorylated protein in *S. pneumoniae* through phosphoproteomic techniques [52]. Furthermore, it has been described that the overexpression and partial depletion of PknB alters cell morphology in *M. tuberculosis* indicating defects in cell wall synthesis and possibly cell division [67]. It has also been shown that PknB from *S. aureus* had a strong regulatory impact on the transcriptional profile of genes encoding proteins involved in the cell wall metabolism [56].

3.4.4. *Transport/binding proteins and lipoproteins*

Different transport proteins were identified as proteins that possibly interact with PrkAc as distinct ABC transporters, and a PTS system involved in the transport of carbohydrates.

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Table 1 – Proteins identified as putative interaction partners of PrkAc classified according to their functional category [a].

Accession #	Protein description	Spot #	Phosphorylation reported [b]
Cell wall			
lmo1548	Similar to cell-shape determining protein MreB	16	–
lmo2525	Similar to MreB-like protein	70	–
lmo1081	Similar to glucose-1-phosphate thymidylyltransferase	40	–
lmo0198	Highly similar to UDP-N-acetylglucosamine pyrophosphorylase (GlmU)	66	Yes
lmo2035	Similar to peptidoglycan synthesis enzymes, putative phospho-N-acetylmuramoyl-pentapeptide-transferase (MurG)	67	–
Transport/binding proteins and lipoproteins			
lmo2372	Similar to ABC transporter, ATP-binding protein	44	–
lmo2415	Similar to ABC transporter, ATP-binding protein	41	–
lmo1849	Similar to metal cations ABC transporter, ATP-binding protein	23	–
lmo2192	Similar to oligopeptide ABC transporter, ATP-binding protein	22, 76	–
lmo2114	Similar to ABC transporter, ATP-binding protein	73	–
lmo0096	Similar to PTS system, mannose-specific, factor IIAB	2, 37	–
Membrane bioenergetics			
lmo2529	Highly similar to H^+-transporting ATP synthase chain beta	30	Yes
lmo2389	Similar to NADH dehydrogenase	18	
Protein secretion			
lmo2510	Translocase binding subunit, SecA	7	Yes
Metabolism of carbohydrates and related molecules — specific pathways			
lmo1581	Acetate kinase (ackA)	14	Yes
lmo1634	Similar to alcohol-acetaldehyde dehydrogenase	28, 63	–
lmo0811	Similar to carbonic anhydrase	49	–
lmo0727	Similar to L-glutamine-D-fructose-6-phosphate amidotransferase	54	–
lmo2556	Similar to fructose-1,6-bisphosphate aldolase (fbaA)	3, 43	Yes
lmo0210	L-lactate dehydrogenase (ldh)	37	Yes
lmo1570	Highly similar to pyruvate kinase (pykA)	52, 53, 56	Yes
lmo0982	Similar to glucanase and peptidase	69	–
Metabolism of carbohydrates and related molecules — main glycolytic pathways			
lmo1054	Highly similar to pyruvate dehyrogenase (dihydrolipoamide acetyltransferase E2 subunit) (pdhC)	5	Yes
lmo1055	Highly similar to dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase complex (pdhD)	1	Yes
lmo2455	Highly similar to enolase (eno)	10	Yes
lmo2459	Highly similar to glyceraldehyde-3-phosphate dehydrogenase (gap)	15, 16, 33	Yes
lmo1072	Highly similar to pyruvate carboxylase (pycA)	9	Yes
lmo1052	Highly similar to pyruvate dehydrogenase (E1 alpha subunit) (pdhA)	19	Yes
lmo1053	Highly similar to pyruvate dehydrogenase (E1 beta subunit) (pdhB)	13	Yes
lmo1374	Similar to branched-chain alpha-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase)	65	Yes
Metabolism of amino acids and related molecules			
lmo0978	Similar to branched-chain amino acid aminotransferase	34	–
lmo1928	Similar to chorismate synthase (aroF)	36	–
lmo0223	Highly similar to cysteine synthase (cysK)	72	Yes
Metabolism of nucleotides and nucleic acids			
lmo2758	Similar to inosine-monophosphate dehydrogenase (guaB)	21, 61	Yes
lmo2559	CTP synthetase (pyrG)	60	–
Metabolism of lipids			
lmo1809	Similar to plsX protein involved in fatty acid/phospholipid synthesis	68	–
lmo1572	Highly similar to acetyl CoA carboxylase (alpha subunit) (accA)	71	–
lmo0970	Similar to enoyl-acyl-carrier protein reductase	75	Yes
Metabolism of coenzymes and prosthetic groups			
lmo0662	Highly similar to phosphomethylpyrimidine kinase thiD	55	–

(continued on next page)

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t1.62 **Table 1** (*continued*)

t1.63 Accession #	Protein description	Spot #	Phosphorylation reported [b]
t1.64 *DNA metabolism*			
t1.65 lmo1320	DNA polymerase III PolC (alpha subunit)	59	–
t1.66 lmo1398	Recombination protein recA	32	–
t1.67			
t1.68 *RNA metabolism*			
t1.69 lmo2072	Similar to redox-sensing transcriptional repressor Rex	29	–
t1.70 lmo2606	DNA-directed RNA polymerase subunit alpha (rpoA)	31	Yes
t1.71 lmo0258	DNA-directed RNA polymerase subunit beta (rpoB)	58	Yes
t1.72 lmo1822	Similar to RNA-binding Sun protein	64	–
t1.73			
t1.74 *Protein metabolism – synthesis – ribosomal proteins*			
t1.75 lmo1658	30S ribosomal protein S2, rpsB	20, 35	–
t1.76 lmo2626	30S ribosomal protein S3, rpsC	24	–
t1.77 lmo2620	50S ribosomal protein L5, rplE	25, 27	Yes
t1.78 lmo2617	50S ribosomal protein L6, rplF	26	–
t1.79 lmo0250	50S ribosomal protein L10, rplJ	81	–
t1.80			
t1.81 *Protein metabolism – synthesis – aminoacyl-tRNA synthetases*			
t1.82 lmo2019	Isoleucyl-tRNA synthetase (ileS)	8	Yes
t1.83 lmo1222	Phenylalanyl-tRNA synthetase beta subunit (pheT)	7	–
t1.84			
t1.85 *Protein metabolism – synthesis – initiation, elongation*			
t1.86 lmo1325	Highly similar to translation initiation factor IF-2 (infB)	62	Yes
t1.87 lmo2654	Highly similar to translation elongation factor G, (fus)	6	Yes
t1.88 lmo2653	Elongation factor Tu (tufA)	11, 12, 42	Yes
t1.89			
t1.90 *Protein metabolism — modification*			
t1.91 lmo1709	Similar to methionine aminopeptidase	74	–
t1.92			
t1.93 *Protein metabolism — folding*			
t1.94 lmo1473	Class I heat-shock protein (molecular chaperone) DnaK	4	Yes
t1.95			
t1.96 *Adaptation to atypical conditions and detoxification*			
t1.97 lmo1138	Similar to ATP-dependent Clp protease proteolytic component	78, 79	Yes
t1.98 lmo1583	Similar to thiol peroxidase	45	Yes
t1.99 lmo1439	Superoxide dismutase (sod)	80	Yes
t1.100			
t1.101 *Similar to unknown proteins*			
t1.102 lmo1401	Hypothetical protein	74	–
t1.103 lmo0799	Hypothetical protein	77	Yes

t1.104 [a] Functional categorization obtained from http://genolist.pasteur.edu.fr/ListiList

[b] Phosphorylation reported in homologous proteins from *Corynebacterium glutamicum* [56], *Bacillus subtilis* [57–59], *Escherichia coli* [60], Q2
Mycobacterium tuberculosis [61,77], *Pseudomonas putida* and *P. aeruginosa* [62], *Lactococcus lactis* [63], *Streptococcus pneumoniae* [64] and *Campylobacter*
t1.105 *jejuni* [65].

Through phosphoproteomic studies, various PTS systems were found phosphorylated at Ser and/or Thr residues in *E. coli* and *L. lactis* [48,51].

3.4.5. *Proteins involved in adaptation to atypical conditions and detoxification*

The protein similar to ATP-dependent Clp protease proteolytic component, classified as a protein implicated in the adaptation to atypical conditions, and the proteins involved in detoxification, superoxide dismutase and thiol peroxidase were identified as putative interactors of PrkAc. All of these proteins were found phosphorylated in other organisms [48,51]. Particularly, it was reported that the activity of the superoxide dismutase from *L. monocytogenes* is regulated by phosphorylation at Ser and Thr residues being most active at its non-phosphorylated form [70].

In summary, in the present work we identify 62 candidates that provide a starting point for further biochemical and cellular studies. The physiological relevance of the proteins and protein families identified in this interactome analysis has to be further examined. According to recent proteomic meta-analysis many of these proteins families (including glycolytic enzymes and elongation factors) are frequently detected as differentially expressed in various conditions raising concern about their specificity [54,55]. Based on previous reports we can hypothesize that some of these frequently identified proteins present in

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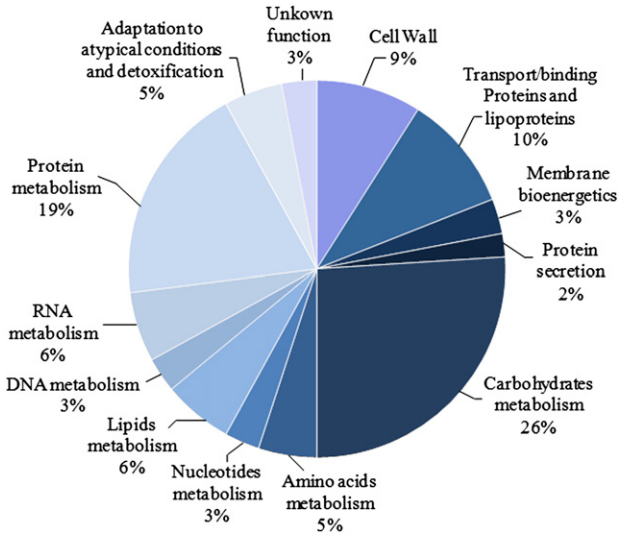

Fig. 8 – Functional classification of the PrkAc putative interactors.

PrkAc interactome may be relevant and should not be excluded without additional analysis. For example, EF-Tu and superoxide dismutase from *L. monocytogenes* have been reported to be phosphorylated *in vivo* in Thr and Ser residues and EF-Tu has been identified as a substrate of STPP in this bacterium [16,70].

4. Conclusions

In this work we describe for the first time a functional STPK from *L. monocytogenes* and start to unravel the processes controlled by protein phosphorylation in this human pathogen. We demonstrated that PrkA is an active STPK able to phosphorylate the exogenous substrate MBP at Ser and/or Thr residues and able to autophosphorylate specific Thr residues within its activation loop sequence. Moreover, using an interactomic approach we identified 62 proteins as potential interaction partners of PrkAc. The diversity of proteins identified suggests that the signal transduction pathways mediated by this STPK in *L. monocytogenes* may affect a large variety of fundamental biological functions including protein synthesis, cell wall metabolism, and carbohydrates metabolism. Interestingly, these processes are also regulated by phosphorylation in other bacteria, suggesting that these enzymes could be controlling conserved functions in prokaryotes [13,15,16,51].

In addition some of the proteins identified in this study arise as possible physiologically relevant interactors of PrkA. In particular evidence coming from other organisms suggests that the enzyme UDP-N-acetylglucosamine pyrophosphorylase (GlmU) implicated in peptidoglycan biosynthesis might be important in PrkA signal transduction pathways. STPKs with extracellular PASTA domains have been reported to bind peptidoglycan fragments and to participate in the regulation of cell wall synthesis and cell division in several bacteria [31]. In addition, phosphorylated residues have been identified in GlmU homologs by phosphoproteomic studies, suggesting that this activity is controlled by the action of STPKs [52]. Interestingly enough, the kinase reported to phosphorylate GlmU in *M. tuberculosis* is PknB, an enzyme highly homologous to PrkA [68].

Also it is worth mentioning the identification of the RNA binding protein Sun as an interactor of PrkA. The specific recovery of this protein, which is expressed at low levels (not identified previously in 2D gels of total protein extracts from *L. monocytogenes*), and co-localized with this kinase in the same operon points to the biological relevance of this interaction. Further work is now being undertaken to validate and characterize these interaction partners of PrkAc and its possible biological relevance.

The present work provided us useful information regarding selected pathways that may be regulated by kinase activity. This framework will be the starting point for a more detailed and comprehensive analysis of the role of this STPK in bacterial physiopathology.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.03.005.

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REFERENCES

[1] Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, Gonzalez-Zorn B, Wehland J, Kreft J. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev 2001;14:584–640.
[2] Gandhi M, Chikindas ML. *Listeria*: a foodborne pathogen that knows how to survive. Int J Food Microbiol 2007;113:1–15.
[3] Hamon M, Bierne H, Cossart P. *Listeria monocytogenes*: a multifaceted model. Nat Rev Microbiol 2006;4:423–34.
[4] Buchrieser C, Rusniok C, Kunst F, Cossart P, Glaser P. *Listeria* Consortium. Comparison of the genome sequences of *Listeria monocytogenes* and *Listeria innocua*: clues for evolution and pathogenicity. FEMS Immunol Med Microbiol 2003;35:207–13.
[5] Hunter T. Signaling—2000 and beyond. Cell 2000;100:113–27.
[6] Cohen P. The origins of protein phosphorylation. Nat Cell Biol 2002;4:E127–30.
[7] Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. Annu Rev Biochem 2000;69:183–215.
[8] Hanks SK, Hunter T. Protein kinases 6. The eukarytic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J 1995;9:576–96.
[9] Bakal CJ, Davies JE. No longer an exclusive club: eukaryotic signaling domains in bacteria. Trends Cell Biol 2000;10:32–8.

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[10] Av-Gay Y, Everett M. The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. Trends Microbiol 2000;8:238–44.

[11] Cozzone AJ. Role of protein phosphorylation on serine/threonine and tyrosine in the virulence of bacterial pathogens. J Mol Microbiol Biotechnol 2005;9:198–213.

[12] Kristich CJ, Wells CL, Dunny GM. A eukaryotic-type Ser/Thr kinase in *Enterococcus faecalis* mediates antimicrobial resistance and intestinal persistence. Proc Natl Acad Sci 2007;104:3508–13.

[13] Molle V, Kremer L. Division and cell envelope regulation by Ser/Thr phosphorylation: *Mycobacterium* shows the way. Mol Microbiol 2010;75:1064–77.

[14] Ohlsen K, Donat S. The impact of serine/threonine phosphorylation in *Staphylococcus aureus*. Int J Med Microbiol 2010;300:137–41.

[15] Wehenkel A, Bellinzoni M, Grana M, Duran R, Villarino A, Fernandez P, Andre-Leroux G, England P, Takiff H, Cerveñansky C, Cole ST, Alzari PM. Mycobacterial Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. Biochim Biophys Acta 2008;1784:193–202.

[16] Archambaud C, Gouin E, Pizarro-Cerda J, Cossart P, Dussurget O. Translation elongation factor EF-Tu is a target for Stp, a serine-threonine phosphatase involved in virulence of *Listeria monocytogenes*. Mol Microbiol 2005;56:383–96.

[17] Sambrook JE, Fritsch F, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.

[18] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

[20] Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol 2002;20:261–8.

[21] Hellman U. Sample preparation by SDS-PAGE and in-gel digestion. In: Jollès P, Jörnvall H, editors. Proteomics in functional genomics. Protein structure analysis. Basel, Switzerland: Birkhäuser Verlag; 2000. p. 43–54.

[22] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.

[23] Boitel B, Ortiz-Lombardía M, Durán R, Pompeo F, Cole ST, Cerveñansky C, Alzari PM. PknB kinase activity is regulated by phosphorylation in two Thr residues and de-phosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. Mol Microbiol 2003;49:1493–508.

[24] Débarbouillé M, Dramsi S, Dussurget O, Nahori MA, Vaganay E, Jouvion G, Cozzone A, Msadek T, Duclos B. Characterization of a serine/threonine kinase involved in virulence of *Staphylococcus aureus*. J Bacteriol 2009;191:4070–81.

[25] Madec E, Laszkiewicz A, Iwanicki A, Obuchowski M, Séror S. Characterization of a membrane-linked Ser/Thr protein kinase in *Bacillus subtilis*, implicated in developmental processes. Mol Microbiol 2002;46:571–86.

[26] Mukhopadhyay S, Kapatral V, Xu W, Chakrabarty AM. Characterization of a Hank's type serine/threonine kinase and serine/threonine phosphoprotein phosphatase in *Pseudomonas aeruginosa*. J Bacteriol 1999;181:6615–22.

[27] Rajagopal L, Clancy A, Rubens CE. A eukaryotic type serine/threonine kinase and phosphatase in *Streptococcus agalactiae* reversibly phosphorylate an inorganic pyrophosphatase and affect growth, cell segregation, and virulence. J Biol Chem 2003;278:14429–41.

[28] Beltramini AM, Mukhopadhyay CD, Pancholi V. Modulation of cell wall structure and antimicrobial susceptibility by a *Staphylococcus aureus* eukaryote-like serine/threonine kinase and phosphatase. Infect Immun 2009;77:1406–16.

[29] Gaidenko TA, Kim TJ, Price CW. The PrpC serine-threonine phosphatase and PrkC kinase have opposing physiological roles in stationary-phase *Bacillus subtilis* cells. J Bacteriol 2002;184:6109–14.

[30] von Heijne G. Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule. J Mol Biol 1992;225:487–94.

[31] Shah IM, Laaberki MH, Popham DL, Dworkin J. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell 2008;135:486–96.

[32] Yeats C, Finn RD, Bateman A. The PASTA domain: a beta-lactam-binding domain. Trends Biochem Sci 2002;27:438–40.

[33] Echenique J, Kadioglu A, Romao S, Andrew PW, Trombe MC. Protein serine/threonine kinase StkP positively controls virulence and competence in *Streptococcus pneumoniae*. Infect Immun 2004;72:2434–7.

[34] Fiuza M, Canova MJ, Zanella-Cléon I, Becchi M, Cozzone AJ, Mateos LM, Kremer L, Gil JA, Molle V. From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division. J Biol Chem 2008;283:18099–112.

[35] Madec E, Stensballe A, Kjellström S, Cladière L, Obuchowski M, Jensen ON, Séror SJ. Mass spectrometry and site-directed mutagenesis identify several autophosphorylated residues required for the activity of PrkC, a Ser/Thr kinase from *Bacillus subtilis*. J Mol Biol 2003;330:459–72.

[36] Durán R, Villarino A, Bellinzoni M, Wehenkel A, Fernandez P, Boitel B, Cole ST, Alzari PM, Cerveñansky C. Conserved autophosphorylation pattern in activation loops and juxtamembrane regions of *Mycobacterium tuberculosis* Ser/Thr protein kinases. Biochem Biophys Res Commun 2005;333:858–67.

[37] D'Ambrosio C, Salzano AM, Arena S, Renzone G, Scaloni A. Analytical methodologies for the detection and structural characterization of phosphorylated proteins. J Chromatogr B Analyt Technol Biomed Life Sci 2007;849:163–80.

[38] Canova MJ, Veyron-Churlet R, Zanella-Cleon I, Cohen-Gonsaud M, Cozzone AJ, Becchi M, Kremer L, Molle V. The *Mycobacterium tuberculosis* serine/threonine kinase PknL phosphorylates Rv2175c: mass spectrometric profiling of the activation loop phosphorylation sites and their role in the recruitment of Rv2175c. Proteomics 2008;8:5215–33.

[39] Johnson DL, Mahony JB. *Chlamydophila pneumoniae* PknD exhibits dual acid specificity and phosphorylates Cpn0712, a putative type III secretion YscD homolog. J Bacteriol 2007;189:7549–55.

[40] Kumar P, Kumar D, Parikh A, Rananaware D, Gupta M, Singh Y, Nandicoori VK. The *Mycobacterium tuberculosis* protein kinase K modulates activation of transcription from the promoter of mycobacterial monooxygenase operon through phosphorylation of the transcriptional regulator VirS. J Biol Chem 2009;284:11090–9.

[41] Meyer HE, Hoffmann-Posorske E, Korte H, Heilmeyer Jr LM. Sequence analysis of phosphoserine-containing peptides. Modif picomolar sensitivity FEBS Lett 1986;204:61–6.

[42] Huse M, Kuriyan J. The conformational plasticity of protein kinases. Cell 2002;109:275–82.

[43] Villarino A, Duran R, Wehenkel A, Fernandez P, England P, Brodin P, Cole ST, Zimny-Arndt U, Jungblut PR, Cerveñansky C, Alzari PM. Proteomic identification of *M. tuberculosis* protein kinase substrates: PknB recruits GarA, a FHA domain-containing protein, through activation loop-mediated interactions. J Mol Biol 2005;29:953–63.

[44] Bendt AK, Burkovski A, Schaffer S, Bott M, Farwick M, Hermann T. Towards a phosphoproteome map of *Corynebacterium glutamicum*. Proteomics 2003;3:1637–46.

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[45] Eymann C, Becher D, Bernhardt J, Gronau K, Klutzny A, Hecker M. Dynamics of protein phosphorylation on Ser/Thr/Tyr in *Bacillus subtilis*. Proteomics 2007;7:3509–26.
[46] Lévine A, Vannier F, Absalon C, Kuhn L, Jackson P, Scrivener E, Labas V, Vinh J, Courtney P, Garin J, Séror SJ. Analysis of the dynamic *Bacillus subtilis* Ser/Thr/Tyr phosphoproteome implicated in a wide variety of cellular processes. Proteomics 2006;6:2157–73.
[47] Macek B, Mijakovic I, Olsen JV, Gnad F, Kumar C, Jensen PR, Mann M. The serine/threonine/tyrosine phosphoproteome of the model bacterium *Bacillus subtilis*. Mol Cell Proteomics 2007;6:697–707.
[48] Macek B, Gnad F, Soufi B, Kumar C, Olsen JV, Mijakovic I, Mann M. Phosphoproteome analysis of *E. coli* reveals evolutionary conservation of bacterial Ser/Thr/Tyr phosphorylation. Mol Cell Proteomics 2008;7:299–307.
[49] Prisic S, Dankwa S, Schwartz D, Chou MF, Locasale JW, Kang CM, Bemis G, Church GM, Steen H, Husson RN. Extensive phosphorylation with overlapping specificity by *Mycobacterium tuberculosis* serine/threonine protein kinases. Proc Natl Acad Sci 2010;20(107):7521–6.
[50] 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: 2764–75.
[51] Soufi B, Gnad F, Jensen PR, Petranovic D, Mann M, Mijakovic I, Macek B. The Ser/Thr/Tyr phosphoproteome of *Lactococcus lactis* IL1403 reveals multiply phosphorylated proteins. Proteomics 2008;8:3486–93.
[52] Sun X, Ge F, Xiao CL, Yin XF, Ge R, Zhang LH, He QY. Phosphoproteomic analysis reveals the multiple roles of phosphorylation in pathogenic bacterium *Streptococcus pneumoniae*. J Proteome Res 2010;9:275–82.
[53] Voisin S, Watson DC, Tessier L, Ding W, Foote S, Bhatia S, Kelly JF, Young NM. The cytoplasmic phosphoproteome of the Gram-negative bacterium *Campylobacter jejuni*: evidence for modification by unidentified protein kinases. Proteomics 2007;7:4338–48.
[54] Petrak J, Ivanek R, Toman O, Cmejla R, Cmejlova J, Vyoral D, Zivny J, Vulpe CD. Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. Proteomics 2008;8:1744–9.
[55] Wang P, Bouwman FG, Mariman EC. Generally detected proteins in comparative proteomics—a matter of cellular stress response? Proteomics 2009;9:2955–66.
[56] Donat S, Streker K, Schirmeister T, Rakette S, Stehle T, Liebeke M, Lalk M, Ohlsen K. Transcriptome and functional analysis of the eukaryotic-type serine/threonine kinase PknB in *Staphylococcus aureus*. J Bacteriol 2009;191:4056–69.
[57] Bott M. Offering surprises: TCA cycle regulation in *Corynebacterium glutamicum*. Trends Microbiol 2007;15:417–25.
[58] O'Hare HM, Durán R, Cerveñansky C, Bellinzoni M, Wehenkel AM, Pritsch O, Obal G, Baumgartner J, Vialaret J, Johnsson K, Alzari PM. Regulation of glutamate metabolism by protein kinases in mycobacteria. Mol Microbiol 2008;70:1408–23.
[59] Durocher D, Jackson SP. The FHA domain. FEBS Lett 2002;513: 58–66.
[60] Absalon C, Obuchowski M, Madec E, Delattre D, Holland IB, Séror SJ. CpgA, EF-Tu and the stressosome protein YezB are substrates of the Ser/Thr kinase/phosphatase couple, PrkC/ PrpC, in *Bacillus subtilis*. Microbiology 2009;155:932–43.
[61] Dasgupta A, Datta P, Kundu M, Basu J. The serine/threonine kinase PknB of *Mycobacterium tuberculosis* phosphorylates PBPA, a penicillin-binding protein required for cell division. Microbiology 2006;152:493–504.
[62] Molle V, Kremer L, Girard-Blanc C, Besra GS, Cozzone AJ, Prost JF. An FHA phosphoprotein recognition domain mediates protein EmbR phosphorylation by PknH, a Ser/Thr protein kinase from *Mycobacterium tuberculosis*. Biochemistry 2003;42:15300–9.
[63] Molle V, Soulat D, Jault JM, Grangeasse C, Cozzone AJ, Prost JF. Two FHA domains on an ABC transporter, Rv1747, mediate its phosphorylation by PknF, a Ser/Thr protein kinase from Mycobacterium tuberculosis. FEMS Microbiol Lett 2004;15:215–23.
[64] Gaidenko TA, Kim TJ, Price CW. The PrpC serine-threonine phosphatase and PrkC kinase have opposing physiological roles in stationary-phase *Bacillus subtilis* cells. J Bacteriol 2002;184:6109–14.
[65] Nováková L, Sasková L, Pallová P, Janecek J, Novotná J, Ulrych A, Echenique J, Trombe MC, Branny P. Characterization of a eukaryotic type serine/threonine protein kinase and protein phosphatase of *Streptococcus pneumoniae* and identification of kinase substrates. FEBS J 2005;272:1243–54.
[66] Nováková L, Bezousková S, Pompach P, Spidlová P, Sasková L, Weiser J, Branny P. Identification of multiple substrates of the StkP Ser/Thr protein kinase in *Streptococcus pneumoniae*. J Bacteriol 2010;192:3629–38.
[67] Kang CM, Abbott DW, Park ST, Dascher CC, Cantley LC, Husson RN. The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. Genes Dev 2005;19:1692–704.
[68] Parikh A, Verma SK, Khan S, Prakash B, Nandicoori VK. PknB-mediated phosphorylation of a novel substrate, N-acetylglucosamine-1-phosphate uridyltransferase, modulates its acetyltransferase activity. J Mol Biol 2009;386: 451–64.
[69] Thakur M, Chakraborti PK. GTPase activity of mycobacterial FtsZ is impaired due to its transphosphorylation by the eukaryotic-type Ser/Thr kinase, PknA. J Biol Chem 2006;281: 40107–13.
[70] Archambaud C, Nahori MA, Pizarro-Cerda J, Cossart P, Dussurget O. Control of *Listeria* superoxide dismutase by phosphorylation. J Biol Chem 2006;281:31812–22.

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