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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.

JOURNAL OF PROTEOMICS XX (2011) XXX-XXX



Serine/threonine protein kinase PrkA of the human pathogen Listeria monocytogenes: Biochemical characterization and

- identification of interacting partners through
- proteomic approaches
- 5 Analía Lima^a, Rosario Durán^a, Gustavo Schujman^b, María Julia Marchissio^b,
- ⁶ María Magdalena Portela^a, Gonzalo Obal^c, Otto Pritsch^{c,d}
- ⁷ Diego de Mendoza^b, Carlos Cerveñansky^{a,*}

⁸ ^aInstitut Pasteur de Montevideo/Instituto de Investigaciones Biológicas Clemente Estable,

9 Unidad de Bioquímica y Proteómica Analíticas, Uruguay

- ¹⁰ ^bInstituto de Biología Molecular y Celular de Rosario (IBR-CONICET) and Departamento de Microbiología,
- 11 Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina
- ¹² ^cInstitut Pasteur de Montevideo, Unidad de Biofísica de Proteínas, Uruguay
- 13 ^dUniversidad 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/Thrphosphatase (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 His6-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.

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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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49 **1.** Introduction

Listeria monocytogenes is a Gram positive rod-shaped bacterium 50that can be recovered from a wide range of sources such as 5152soil, water, vegetation, effluents, human and animal feces and 53fresh and processed foods. This bacterium can tolerate hostile and stress conditions as high salt concentrations, acid pH and can 5455grow at temperatures ranging from -1 °C to 45 °C [1]. These 56features allow these bacteria to survive many of the strategies 57used for food preservation and thus they become an important threat for human health. As a result, L. monocytogenes arises as an 5859important foodborn pathogen, etiologic agent of listeriosis, and a sporadic but very serious disease [2]. Pregnant women, newborns, 60 61 elderly and immunosuppressed individuals have predisposition to more severe presentation of the disease. In these high-risk 62 populations, listeriosis can produce very serious clinical mani-63 64 festations like septicemia, meningitis, meningoencephalitis and abortions, resulting in death in 20-30% of the cases despite early 65 antibiotic treatment [1]. Pathogenesis of L. monocytogenes is 66 mediated by its ability to effectively invade and replicate within 67 a broad range of eukaryotic cells and to cross the intestinal barrier, 68 blood-brain barrier, and plancental barrier in the mammalian 69 70host. L. monocytogenes has a relatively complex infectious cycle 71 with different stages: internalization in host cells, intracellular 72proliferation and intercellular spread. Each stage of the intracel-73 lular parasitism is dependent upon the differential expression of 74 distinct virulence factors [3].

75 The extraordinary capacity of L. monocytogenes to adapt and 76respond to environmental changes seems to be related to an 77 extensive repertoire of predicted regulatory proteins, including different RNA polymerase sigma factors, transcription factors and 78 protein phosphorylation systems [4]. Protein phosphorylation is a 79major mechanism in signal transduction processes by which 80 environmental stimuli are translated into cellular responses and 81 represents one of the most important post-translational mod-82 ifications regulating enzyme activities and protein interactions 83 [5,6]. Signal transduction in prokaryotes is predominantly accom-84 85 plished by the so called two-component systems, consisting of His-kinase sensors and their associated response regulators [7]. In 86 contrast, in eukaryotes such signaling pathways are mainly 87 carried out by Ser/Thr or Tyr-kinases [8]. Long time thought to be 88 exclusive to eukaryotes, a bulk of evidence raised from genome 89 90 sequence data now indicates that Ser, Thr, and Tyr phosphory-91 lation is also widespread in prokaryotes [9]. These eukaryotic-like 92signaling systems have been shown to control essential processes in bacteria, including development, cell growth, stress responses, 93 central and secondary metabolism, biofilm formation, antibiotic 94resistance, and virulence [9-15]. In the case of L. monocytogenes the 95presence of eukaryotic-like phosphorylation systems has been 96 predicted by genome analysis. In particular, it was reported that 97 the stp gene (lmo1821) encodes a functional Ser/Thr protein 98 phosphatase (STPP) required for growth of L. monocytogenes and 99 100 virulence in murine model of infection. In addition, the elongation 101 factor EF-Tu was described as a target for this phosphatase [16]. However, there is no information regarding the corresponding 102 phosphorylating enzymes, endogenous substrates and their role 103in bacteria physiology and physiopathology. 104

105 In the present work we report the cloning, expression and 106 purification of the catalytic domain of the gene product of Imo1820, named PrkA, a putative transmembrane Ser/Thr 107 protein kinase (STPK) coded by the *L. monocytogenes* genome. 108 We produced the catalytic domain of PrkA (PrkAc) as a 109 functional enzyme able to phosphorylate an exogenous sub-110 strate at Ser and/or Thr residues. We also demonstrate that 111 PrkAc is autophophorylated at specific conserved Thr residues. 112 Finally, as a first attempt in deciphering the potential role of 113 PrkA, we identified 62 proteins that possibly interact, directly or 114 indirectly, with the phosphorylated catalytic domain. These 115 putative interaction partners participate in a wide range of 116 cellular processes, indicating that PrkA could have a role in the 117 regulation of a diversity of essential biological functions in 118 *L. monocytogenes.* 119

2.1. Bacterial strains, vectors, and culture conditions 122

Escherichia coli DH5 α and E. coli M15[pREP] (Qiagen) were used 123 for plasmid maintenance and protein expression, respective-124 ly. The plasmid pQE32 (Qiagen) was used as protein expression 125 vector. E. coli strains were cultured on Luria-Bertani (LB) agar or 126 broth. When required, media were supplemented with 100 μ g/ml 127 ampicillin and 25 μ g/ml kanamycin. L. monocytogenes EGDe was 128 cultured on LB agar or broth supplemented with 50 mM glucose. 129

2.2. General genetic techniques 130

Genomic DNA from *L. monocytogenes* EGDe was prepared by 131 heating bacterial colonies in ultrapure water at 100 °C for 5 min. 132 Cellular debris were discarded by centrifugation a 10,000 *g* and 133 the supernatant, containing genomic DNA, was used as 134 template for PCR reactions. Plasmid DNA from *E. coli* cells was 135 prepared with Wizard Plus Minipreps DNA purification system 136 (Promega). DNA fragments from agarose gels were obtained 137 using the GFX PCR DNA and Gel Band Purification Kit (GE 138 Healthcare). DNA digestion with restriction enzymes, ligation 139 reactions with T4 DNA ligase and agarose gel electrophoresis 140 were carried out according to methods described by Sambrook 141 et al. [17]. Transformation of *E. coli* competent cells with plasmid 142 DNA was performed using the CaCl₂ method [17]. 143

2.3. Sequence analysis 144

Protein sequence of the potential STPK PrkA (*lmo1820*) from 145 *L. monocytogenes* EGDe was obtained from Listilist web site (http:// 146 genolist.pasteur.fr/ListiList/). Multiple sequence alignment of 147 PrkA with other characterized STPKs from related microorganism 148 was carried out using ClustalW software (http://www.ebi.ac.uk/ 149 Tools/clustalw/). Analyses related to sequence conservation were 150 performed using the Genedoc software http://www.nrbsc.org/gfx/ 151 genedoc/. Other bioinformatics tools (TMHMM server v 2.0, 152 RADAR available at http://www.expasy.ch/tools/) were used for 153 the prediction of transmembrane domains and sequence repeats. 154

2.4. Cloning, expression and purification of PrkAc 155

PrkAc (amino acids 1–338) was produced as a His_6-tagged $_{156}$ protein in E. coli. For that purpose, DNA fragment corresponding $_{157}$

to PrkAc was synthesized using genomic DNA from L. mono-158 cytogenes EGDe as a template and the following primers: 1820CU, 1595'-GATGCTGGATCCTGATTGGTAAGCGATT-3' and 1820CL, 5'-160 AACAATGTCGACCTATTTCTTTTTTTTTGCTCAT-3'. Primers 161 1820CU and 1820CL contained the BamHI and SalI restrictions 162sites, respectively. After digestions with the corresponding 163 restriction enzymes, the PCR product was cloned into pQE32 164 vector (Qiagen). The resulting plasmid was introduced into E. coli 165166 M15[pREP4] for protein expression. The sequence of the cloned protein was verified by DNA sequencing. 167

The expression strain was grown at 37 °C until mid-log phase 168 in LB broth supplemented with ampicillin and kanamycin. 169Induction of protein expression was conducted for 4 h at 37 °C 170after the addition of 1 mM isopropyl- β -thiogalactopyranoside. 171 Then, bacterial pellets were resuspended in 50 mM NaH₂PO₄, 172300 mM NaCl, 10 mM imidazol and lysed by sonication on ice 173followed by centrifugation. The His6-tagged proteins were 174purified under native condition by Ni²⁺-affinity chromatography 175according to the manufacturer instruction (Qiagen) followed by 176dialysis against 50 mM HEPES, pH 7.2. Protein purification was 177 monitored by SDS-PAGE [18] and protein concentrations were 178 determined by Bradford assays [19]. 179

180 2.5. In vitro phosphorylation and de-phosphorylation181 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 MnCl₂, and 100 μ M ATP. Myelin basic protein (MBP) was used as substrate at a concentration of 25 μ M (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 Ni²⁺-affinity resin and incubated at 37 °C in presence of MnCl₂, ATP as described above. Autophosphosphorylated peptides were detected by MS after tryptic digestion.

196 2.6. Sample preparation for MS analysis

Proteolytic digestion was carried out by incubating the proteins 197with trypsin (sequence grade, Promega) in 50 mM ammonium 198 bicarbonate, pH 8.3, for 2 h at 37 °C (enzyme-substrate ratios 199 1:10). The β -elimination reactions at phosphoresidues were 200performed by treating 2 µg of PrkAc tryptic peptides with a 201saturated solution of Ba(OH)₂ at room temperature for 4 h as 202previously reported [20]. Then, the samples were acidified with 20320410% 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% 214 TFA. 215

2.7. MALDI-TOF MS analysis 216

Mass spectra of peptides mixtures were acquired in a 4800 217 MALDI TOF/TOF instrument (Applied Biosystems) in positive 218 ion reflector mode. Mass spectra were externally calibrated 219 using a mixture of peptide standards (Applied Biosystems). 220 MS/MS analyses of selected peptides were performed. 221

Proteins were identified by the database searching of 222 measured peptide m/z values using the MASCOT program 223 (Matrix Science http://www.matrixscience.com/search form 224 select.html), and based on the following search parameters: 225 monoisotopic mass tolerance, 0.05 Da; fragment mass toler- 226 ance, 0.3 Da; partial methionine oxidation, cysteine carbami- 227 domethylation and one missed tryptic cleavage allowed. 228 Protein mass and taxonomy were unrestricted. Significant 229 scores (p<0.05) were used as criteria for positive protein 230 identification. 231

Phosphorylation state of presumptive phosphopeptides 232 was confirmed by MS/MS experiments. The identification of 233 phosphorylated residues was achieved by MS/MS analysis of 234 peptides treated with Ba(OH)₂. 235

2.8. Preparation of L. monocytogenes protein extracts 236

L. monocytogenes were grown in LB supplemented with 50 mM 237 glucose at 37 °C until mid-log phase. Pellets were resuspended 238 in 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-239 100, 1% glycerol, 10 μ g/ml proteases inhibitor mix (GE Health-240 care). Bacterial suspension was treated with 1 mg/ml lysozyme 241 and incubated on ice for 30 min. Then, cells were disrupted by 242 sonication on ice. After treatment with 10 μ g/ml RNAse and 5 μ g/243 ml DNAse, cells debris was removed by centrifugation at 10,000 g 244 for 30 min at 4 °C and the supernatants were collected and 245 stored at -80 °C. Total protein concentration was determined 246 using 2D-Quant kit (GE Healthcare).

2.9. Surface plasmon resonance analysis 248

Surface plasmon resonance experiments were performed on a 249 BIAcore 3000 instrument (BIAcore, Piscataway, NJ). PrkAc was 250 immobilized using standard amine-coupling procedures 251 (Amine Coupling Kit, BIAcore) on a CM5 sensorchip at pH 4 252 to a final density of 8800 resonance units (RU). Then, the 253 instrument was primed with running buffer (20 mM HEPES pH 254 7.4, 150 mM NaCl, 5 mM EDTA, 0.005% Tween 20). A flow cell 255 activated and blocked with ethanolamine was left as a control 256 surface for non-specific binding. 257

Forty microlitres of 15 μ g/ml of a *L. monocytogenes* total 258 protein extract were injected onto the surfaces. Binding 259 experiments were performed at 25 °C at a flow rate of 10 μ l/ 260 min during 240 s. After extensive washing with running 261 buffer, ligands were eluted using 50 μ l of 20 mM glycine pH 3 262 or 1 M NaCl at flow rate of 100 μ l/min during 30 s in two 263 independent experiments. All data processing was carried out 264 using the BIAevaluation 4.1 software provided by BIAcore. 265 Binding responses were first double-referenced by subtracting 266

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

269 2.10. Preparation of immobilized PrkAc affinity resin

Recombinant PrkAc was covalently coupled to HiTrap NHS-270activated HP (Amersham Biosciences), following the instruc-271tions provided by the manufacturer. Briefly, the resin was 272273washed with cold 1 mM HCl and activated with coupling 274buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). Then, 400 μg of PrkAc was added to the activated resin and incubated for 4 h 275at 4 °C with gentle agitation. Washing and blocking of the 276resin unreacted groups was performed by alternated washes 277with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 and 0.1 M 278CH₃COONa, 0.5 M NaCl, pH 4. The same process was carried 279out to prepare a control resin, but omitting the addition of 280PrkAc in the coupling step. 281

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.

287 2.11. Affinity chromatography

288L. monocytogenes protein extract (600 µl, 7 mg/ml) prepared as 289described was added to immobilized PrkAc and control resin 290(previously equilibrated with 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% glycerol) and incubated 291 for 4 h at 4 °C with gentle agitation. Then, resins were 292extensively washed with 10 mM HEPES, 150 mM NaCl, pH 8.3 293and finally bound proteins were eluted with 20 mM glycine pH 2943.0. The chromatographic fractions were analyzed by 12.5% SDS-295PAGE followed by silver staining. Additionally, eluted fractions 296were concentrated and analyzed by 2D electrophoresis. Two 297affinity chromatography experiments were run independently 298with different cell extracts. 299

300 2.12. 2D electrophoresis

First dimension was performed with commercially available 301 302 IPG-strips (7 cm, linear 3-10, GE Healthcare). Eluted protein 303 fractions were purified and concentrated with 2-D Clean-Up kit (GE Healthcare) and dissolved in 125 µl of rehydration solution 304(7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer 3-10 [GE 305 Healthcare], 0.002% bromophenol blue). Samples in rehydration 306 solution were loaded onto IPG-strips by passive rehydration 307 during 12 h at room temperature. 308

The isoelectric focusing was done in an IPGphor Unit 309 (Pharmacia Biotech) employing the following voltage profile: 310 311 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 312 phase of 5000 V to reach total of 6.5 kVh. Prior running the 313 second dimension, IPG-strips were reduced for 15 min in 314 315 equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) supplemented 316 with DTT (10 mg/ml) and subsequently alkylated for 15 min in 317 equilibration buffer supplemented with iodoacetamide 318 (25 mg/ml). The second-dimensional separation was per-319 320 formed in 12.5% SDS-PAGE using a SE 260 mini-vertical gel electrophoresis unit (GE Healthcare). The size markers used321were Amersham Low Molecular Weight Calibration Kit for SDS322Electrophoresis (GE Healthcare).323

The gels were silver stained according to protocols 324 described [22]. Images were digitalized using a UMAX Power- 325 Look 1120 scanner and LabScan 5.0 software (GE Healthcare). 326

3. Results and discussion

3.1. Sequence analysis

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The analysis of the L. monocytogenes EGDe genome revealed the 330 presence of two putative STPKs (Imo0618 and Imo1820) and one 331 STPP (lmo1821). In the 10.2 kbp region that encloses the gene 332 coding PrkA (lmo1820) eight open reading frames are found 333 (http://genolist.pasteur.fr/ListiList/) (Fig. 1). This gene cluster 334 also includes the gene lmo1821 and other genes involved in 335 information pathways (DNA, RNA and protein metabolism 336 and modification) (lmo1819, lmo1822, fmt, and priA) and 337 intermediary metabolism (lmo1818 and lmo1825). The presence 338 in the same genome region of a STPP gene preceding the STPK 339 gene was also found in other bacteria suggesting a functional 340 association between theses enzymes [23-27]. Particularly it has 341 been observed that such STPK/STPP couples act as functional 342 pairs in Mycobacterium tuberculosis, Staphylococcus aureus and 343 Bacillus subtilis [23,25,28,29]. 344

The STPK PrkA is a predicted 655 amino acids transmembrane 345 protein, with a theoretical molecular mass of 72 kDa and a pI 346 value of 4.99. Sequence analysis showed the presence of a 347 pattern of basic residues followed by a predicted transmembrane 348 domain suggesting that the N-terminal region (residues 1-338) is 349 orientated toward the cytoplasm [30]. It was also observed that 350 PrkA N-terminal sequence contains a predicted STPK that 351 exhibits all the conserved subdomanis (subdomains I to V, VIa, 352 VIb and VII to XI) and the nearly invariant residues that define the 353 Hanks family of eukaryotic protein kinases [8] (Fig. 2). Protein 354 sequence alignments showed that the putative kinase domain of 355 PrkA has high homology with the catalytic domain of other well 356 studied bacterial STPK, such as PrkC from B. subtilis (68% identity), 357 StkP form Streptococcus pneumoniae (53% identity), Stk1 from S. 358 aureus (49% identity) and PknB from M. tuberculosis (46% identity) 359 (Fig. 2). 360

Analysis of the C-terminal domain sequence of PrkA showed 361 the presence of several copies of PASTA domains (Penicillin- 362 binding protein and Ser/Thr kinase Associate) (supplementary 363 Fig. 1). This domain interacts with peptidoglycan fragments and 364 β-lactamic antibiotics and is present in high molecular weight 365 penicillin-binding proteins and eukaryotic-like STPKs of a 366 variety of pathogens [31,32]. This structural organization, with 367 extracellular PASTA domains and intracellular kinase domain is 368 also well conserved in different prokaryotic STPKs, including 369 PknB from M. tuberculosis, Corynebacterium glutamicum and 370 S. aureus, PrkC from B. subtilis and StkP from S. penumoniae 371 [23,24,33-35], pointing to the regulation of related processes by 372 protein phosphorylation in response to similar stimuli in these 373 microorganisms. STPKs from this group participate in the 374 regulation of diverse bacterial processes including growth, cell 375 division, developmental states, central and secondary metabo- 376 lism and expression of virulence factors [13–15]. 377

JOURNAL OF PROTEOMICS XX (2011) XXX-XXX

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 <u>putative</u> 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.

378 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 His₆-tagged protein (PrkAc). DNA sequence
corresponding to amino acids 1–338 was amplified by PCR and
partial sequencing assured error-free amplification and inframe fusion with the His₆-tag of the expression vector.

Purification of PrkAc was performed under native conditions 385 using Ni²⁺-NTA affinity resin. SDS-PAGE analysis showed a band 386 that migrates according to the predicted molecular mass of the 387 388 recombinant protein (39 kDa for the catalytic domain) and two 389 additional bands ranging from 41 to 43 kDa (Fig. 3). All these proteins were identified by PMF as PrkA demonstrating that the 390 protein expressed in E. coli has at least three isoforms with 391 different migration behavior in SDS-PAGE. 392

393 The recombinant protein PrkAc was examined for its ability to phosphorylate the exogenous substrate MBP. Comparison 394of mass spectra of digested MBP after and before incubation 395 with PrkAc in the presence of ATP and Mn² revealed that 396 sequence 30-41 is phosphorylated by the kinase. Signal of 397 native sequence (m/z=1339.61) present in control spectra 398 decreased after phosphorylation reaction and concomitantly 399 a signal with a mass increment of 80 Da (m/z = 1419.68) became 400apparent (Fig. 4). This particular MBP peptide was found to be 401 systematically and extensively phosphorylated by several myco-402 bacterial STPKs. Its detection by MS was previously reported as a 403404 sensitive marker of kinase activity [36]. Phosphorylation of MBP tryptic peptide 30-41 by PrkAc was further confirmed by MS/MS 405 analysis (Fig. 4). The presence of daughter ions with mass 406differences of 80 Da (loss of HPO₃) and 98 Da (loss of H_3PO_4) is 407characteristic of phosphorylated peptides [36,37]. These results 408 clearly demonstrate that PrkAc was produced in E. coli as a 409functional STPK able to phosphorylate the exogenous substrate 410MBP. The fact that PrkAc phosphorylates the same MBP peptide 411 than mycobacterial protein kinases probably reflects some 412 specificity of bacterial kinases towards this sequence. 413

414 3.3. Identification of phosphorylated peptides and residues 415 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=3893.90 421 could be assigned to the mono-, di-, and tri-phosphorylated 422 tryptic peptide 160–183 respectively) (Fig. 5). Additionally, the 423 multiple phosphorylated state of these peptides was confirmed 424 by MS/MS analyses (data not shown). It is interesting to note that 425 this multiple phosphorylated peptide is enclosed within the 426 conserved motifs DFG and PE of Hanks kinases corresponding to 427 the activation loop in several STPK from related bacteria 428 [8,23,25,36,38–40].

The identification of phosphorylation sites by MS/MS analyses 430 is usually challenging because fragmentation of phosphopeptides 431 is mainly dominated by the neutral loss of phosphate group. This 432 fact precludes the detection of sequence-specific ion signals 433 rendering difficult the localization of modification sites [36]. For 434 that reason, we treated the phosphorylated peptides with $Ba(OH)_2$ 435 to generate de-hydro amino acids from phospho-Ser and 436 phospho-Thr residues by β -elimination of H₃PO₄. Such deriva- 437 tives have better properties for MS/MS experiments. Moreover 438 they show a mass difference of 18 Da compared to the parent 439 amino acid residue, thus becoming a useful tag for phosphor- 440 esidue identification [41]. The spectrum of Ba(OH)₂ treated 441 peptides showed signals 18, 36 and 54 Da lower than the expected 442 for native peptides 160-183, indicating the presence of species 443 that have been generated by multiple β -elimination of phosphate 444 group (Fig. 6). 445

The phosphorylation sites were assigned by manual inspec- 446 tion of MS/MS spectrum of the ion generated after β-elimination 447 reaction of the tri-phosphorylated peptide. This spectrum 448 shows mostly y-ions and the presence of signals with mass 449 differences of 18 Da (and multiple thereof) in relation to the 450 theoretical expected values, was clearly detected allowing the 451 unequivocally identification of modified residues (Fig. 6). The 452 results allowed us to identify the phosphorylation sites as 453 Thr171, Thr174 and Thr176 within the sequence 160-183 of PrkA 454 activation loop. At least two of this Thr residues are highly 455 conserved in the activation loop sequence of other bacterial 456 STPKs and its phosphorylated state has been reported 457 [23,35,36,38,40]. In addition, it was demonstrated for some 458 STPKs, such as PrkC from B. subtilis and PknB from M. tuberculosis, 459 that the phosphorylation of these conserved Thr residues in the 460 activation loop regulates kinase activity [23,35]. 461

To test if phosphorylation of the activation loop sequence 462 was a result of an autocatalytic reaction, the recombinant kinase 463 was de-phosphorylated using alkaline phosphatase, purified 464 using Ni^{2+} -NTA resin and re-incubated in the presence of ATP 465 and Mn^{2+} . The phosphorylation status of PrkAc was followed by 466

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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 467that phosphatase treatment results in activation loop de-468 phosphorylation, indicated by the disappearance of phosphor-469 ylated species and the increase of native peptide m/z signal. After 470 471 incubation of the de-phosphorylated enzyme with ATP the 472activation loop phosphopeptides were clearly detected in the mass spectrum, indicating that PrkAc presented autocatalytic 473474 activity (data not shown).

The activation loop phosphorylation status is important to 475 control the active/inactive conformational switch in numer-476 ous kinases. A wide range of regulatory mechanism has been 477 suggested for this loop, such as the contribution to the 478 appropriate alignment of the catalytic residues and the 479 correction of the relative orientation of different domains 480 allowing the binding of the protein substrate and/or ATP [42]. 481 The relevance of the activation loop phosphorylation has been 482

JOURNAL OF PROTEOMICS XX (2011) XXX-XXX



Fig. 3 – Over-expression and purification of His₆-tagged PrkAc. Proteins were purified with Ni²⁺-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.

483 demonstrated by using point mutation in PknB from 484 M. tuberculosis and PrkC from B. subtilis [23,35]. In addition 485 our group has demonstrated that phosphorylated residues in the activation loop are not only important for enzyme activity 486 but also defines a high affinity docking site that is relevant for 487 substrate recruitment [43]. Considering these evidences from 488 homologous proteins, we can suggest that the very well 489conserved phosphorylation pattern here reported for PrkA, 490participates in activity control and perhaps also in substrate 491 recruitment by protein interactions mediated by specific 492 phospho-residues recognition. 493

494 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 501PrkAc we carried out affinity chromatography experiments 502using the conditions obtained from surface plasmon resonance 503experiments. For that purposes, we first immobilized recombi-504nant PrkAc to a Hi-trap NHS-activated resin HP (Amersham 505506 Bioscience). A fraction of the resin submitted to the process of immobilization was digested with trypsin and analyzed by MS 507to confirm the coupling of PrkAc. Only tryptic masses from 508PrkAc were detected, discarding the presence of significant 509amounts of contaminating proteins. The incubation of the 510covalently bound kinase with MBP under phosphorylation 511conditions showed that the immobilized protein was an active 512513enzyme (data not shown).

514 To recover either individual proteins or protein complexes 515 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 516 EGDe. After extensive washing the ligands were eluted using 517 acid pH. The different fractions of the affinity chromatography 518 were primarily analyzed by one-dimensional SDS-PAGE and 519 visualized by silver staining. From these analyses we could 520 observed that many proteins were retained by PrkAc resin 521 while we did not detect proteins in control resins (data not 522 shown). 523

In order to achieve a better resolution, eluted protein were 524 separated by 2D electrophoresis. Analysis of 2D gels allowed 525 us to detect a specific protein profile of eluted proteins in 526



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 527profile of total cellular extracts (data not shown). Spots 528529detected in all replicates were processed for protein identification by PMF (Fig. 7 and supplementary Fig. 2). This strategy 530allowed the identification of 62 proteins that possibly interact, 531 directly or indirectly, with PrkAc. For each protein identified, 532supplementary Table 1 reports protein Mascot scores and ion 533scores generated from fragmentation of selected *m*/z values, 534535protein sequence coverage, and other parameters used in the identification. Table 1 displays the complete list of PrkAc 536putative interactors identified in this study, grouped accord-537538 ing to their functional category. The two largest groups were 539 composed of proteins functionally related to the metabolism of carbohydrates (26%) and protein synthesis (19%) (Fig. 8). 540This is followed by proteins involved in transport and binding 541of proteins and lipoproteins (10%) and in cell wall metabolism 542(9%). A primary conclusion that arises from the diversity of 543proteins identified as potential interaction partners of PrkAc 544

could be that the signal transduction pathways mediated by 545 this STPK in L. monocytogenes could be affecting a great variety 546 of fundamental biological functions. 547

Since the immobilized protein is the autophosphorylated 548 catalytic domain of a STPK, we consider the possibility that some 549 of the potential interacting partners were also substrates of the 550 kinase. Therefore we searched reported phosphoproteomes to 551 see if the identified proteins were phosphorylated at Ser or Thr in 552 other microorganism. We found that 48% of the proteins were 553 described to be phosphorylated in at least one of the following 554 microorganisms: C. glutamicum, B. subtilis, E. coli, M. tuberculosis, 555 Pseudomonas aeruginosa, P. putida, Lactococcus lactis, S. pneumoniae, 556 and Campylobacter jejuni [44–53]. 557

It is also important to note that many of these putative 558 partners were reported as the proteins most frequently 559 identified in differential expression proteomic analysis based 560 on 2D gel approaches [54,55]. If the identification of these 561 proteins represents a technical artifact or reveals that they 562 participate in a general cell mechanism is still a matter of 563 debate [54,55]. Even when our experimental approach points 564 to a specific interaction of these proteins with PrkAc, we have 565 to be very careful with the interpretation of these results. In 566 addition to these frequently detected proteins, less abundant 567 regulatory proteins were also identified as possible interactors 568 of PrkAc. 569

The list of proteins and protein families identified provides 570 information regarding possible functions of PrkAc. In the 571 following paragraphs we focus on some of the potential 572 interaction partners of PrkAc that are related to STPKs function 573 in other organisms and whose relevance has been reported or 574 strongly suggested. 575

3.4.1. Proteins involved in the carbohydrate metabolism 576We identified 15, proteins related to the glycolytic pathway and 577 the tricarboxylic acid (TCA) cycle. Some of them (aldolase, 578 glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvate 579 kinase, lactate dehydrogenase, acetate kinase, dihydrolipoamide 580 dehydrogenase and α -cetoglutarate dehydrogenase) were found 581 to be phosphorylated at Ser, Thr or Tyr residues trough 582 phosphoprotemic studies in other microorganisms [44-53]. It 583 was also proved that the transcriptional profile of two enzymes 584 involved in the TCA cycle (dihydrolipoamide succinyltransferase 585 and oxoglutarate dehydrogenase E1) is affected by the STPK PknB 586 from S. aureus [56]. Additionally, in M. tuberculosis and 587 C. glutamicum it has been demonstrated that the regulation of 588 TCA cycle is mediated by STPKs [57,58]. In these bacteria, the 589 STPKs PknB and PknG phosphorylate a protein containing a FHA 590 domain (GarA y OdhI in M. tuberculosis and C. glutamicum 591 respectively) which in their de-phosphorylated forms inhibit 592 the enzyme 2-oxoglutarato dehydrogenase [57,58]. FHA domains 593 are small protein modules that mediate protein-protein inter- 594 actions in the STPK-mediated signal transduction pathways 595 through the recognition of specific phosphorylated residues [59]. 596 Genome sequence analyses have revealed that all members of 597 the order actinomycetales present GarA-homologous proteins 598 which show strong sequence conservation at the C-terminus 599 FHA domain [43]. However, the analysis of the proteins coded by 600 the L. monocytogenes genome does not predict the presence FHA- 601 containing proteins. Therefore, the STPK PrkA in L. monocytogenes 602 could be involved in the modulation of the TCA cycle through a 603

JOURNAL OF PROTEOMICS XX (2011) XXX-XXX



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 theorder 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

600 We faithful the following proteins that the implicated in 609 DNA and RNA synthesis: DNA polymerase, RNA polymerase 610 (α and β subunits), transcriptional repressor Rex and the RNA 611 binding protein Sun. The RNA polymerase was found phos-612 phorylated by phosphoproteomic approaches in M. tuberculosis 613 and S. pneumoniae [49,52].

One of the most interesting proteins arising from this studyis the RNA binding protein Sun. The gene that codes for Sun

(*lmo*1822) is located in the same genomic region and adjacent 616 to the genes *lmo*1820 and *lmo*1821 (coding for PrkA and Stp 617 respectively), probably organized in an operon. This observa- 618 tion suggests that both proteins could be genetically and 619 functionally linked. The fact that both STPK and its substrates 620 are encoded in the same genomic region is recurrent for many 621 STPKs from many organisms [60–63].

We also detected 9 proteins involved in the biosynthesis of 623 proteins, as ribosomal proteins, aminoacyl t-RNA synthetases, 624 the translation initiation factor InfB, and the translation 625 elongation factors EF-Tu and EF-G. The translation initiation 626 and elongation factors and the isoleucyl-tRNA synthetase were 627 found to be phosphorylated in other bacteria [44–46,48–53]. 628

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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.

636 3.4.3. Proteins involved in the cell wall metabolism

In this study we identified 5 proteins that participate in the cell 637 638 wall metabolism: the cell shape determining proteins MreB 639 and Mbl, and the proteins involved in the peptidoglycan synthesis, N-acetylglucosaminyl transferase, UDP-N-acetylglu-640 cosamine pyrophosphorylase (GlmU) and glucose-1-phosphate 641 thymidylyltransferase. Several STPKs, in particular the ones 642 that have PASTA domains as sensor extracellular domains, have 643 been implicated in the regulation of the cell wall metabolism. 644

Different proteins related to the growth and cellular division 645 were identified as substrates of STPKs, as DivA, PbpA, FtsZ and 646 GlmU from *M. tuberculosis* and GlmS from *S. pneumoniae* [61,65–647 69]. GlmU was also found as a phosphorylated protein in 648 *S. pneumoniae* through phosphoproteomic techniques [52]. 649 Furthermore, it has been described that the overexpression 650 and partial depletion of PknB alters cell morphology in 651 *M. tuberculosis* indicating defects in cell wall synthesis and 652 possibly cell division [67]. It has also been shown that PknB from 653 *S. aureus* had a strong regulatory impact on the transcriptional 654 profile of genes encoding proteins involved in the cell wall 655 metabolism [56].

3.4.4. Transport/binding proteins and lipoproteins 657 Different transport proteins were identified as proteins that 658 possibly interact with PrkAc as distinct ABC transporters, and 659 a PTS system involved in the transport of carbohydrates. 660

JOURNAL OF PROTEOMICS XX (2011) XXX-XXX

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	Metabolism of coen	zymes ana prostnetic groups		
ImoU662 Highly similar to phosphomethylpyrimidine kinase thiD 55 –	lmo0662	Highly similar to phosphomethylpyrimidine kinase thiD	55	-

⁽continued on next page)

JOURNAL OF PROTEOMICS XX (2011) XXX-XXX

Accession #	Protein description	Spot #	Phosphorylation reported ^b
DNA metabolism			
lmo1320	DNA polymerase III PolC (alpha subunit)	59	-
lmo1398	Recombination protein recA	32	-
RNA metabolism			
lmo2072	Similar to redox-sensing transcriptional repressor Rex	29	-
lmo2606	DNA-directed RNA polymerase subunit alpha (rpoA)	31	Yes
lmo0258	DNA-directed RNA polymerase subunit beta (rpoB)	58	Yes
lmo1822	Similar to RNA-binding Sun protein	64	-
Protein metabolism	– synthesis – ribosomal proteins		
lmo1658	30S ribosomal protein S2, rpsB	20, 35	-
lmo2626	30S ribosomal protein S3, rpsC	24	-
lmo2620	50S ribosomal protein L5, rplE	25, 27	Yes
lmo2617	50S ribosomal protein L6, rplF	26	-
lmo0250	50S ribosomal protein L10, rplJ	81	-
Protein metabolism	- synthesis - aminoacyl-tRNA synthetases	0	
lmo2019	Isoleucyl-tRNA synthetase (ileS)	8	Yes
lmo1222	Phenylalanyl-tRNA synthetase beta subunit (pheT)	/	-
Protein metabolism	– synthesis – initiation, elongation		
lmo1325	Highly similar to translation initiation factor IF-2 (infB)	62	Yes
lmo2654	Highly similar to translation elongation factor G, (fus)	6	Yes
lmo2653	Elongation factor Tu (tufA)	11, 12, 42	Yes
Protein metabolism	- modification		
lmo1709	Similar to methionine aminopeptidase	74	-
Protein metabolism	— foldina		
lmo1473	Class I heat-shock protein (molecular chaperone) DnaK	4	Yes
Adaptation to atypi	cal conditions and detoxification		
lmo1138	Similar to ATP-dependent Clp protease proteolytic component	78, 79	Yes
lmo1583	Similar to thiol peroxidase	45	Yes
lmo1439	Superoxide dismutase (sod)	80	Yes
Similar to unbrown	wataina		
Similar to unknown	Unatherical protein	74	
11101401	nypotiletical protein	/4	_

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],

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].

664 3.4.5. Proteins involved in adaptation to atypical conditions 665 and detoxification

666 The protein similar to ATP-dependent Clp protease proteolytic 667 component, classified as a protein implicated in the adapta-668 tion to atypical conditions, and the proteins involved in 669 detoxification, superoxide dismutase and thiol peroxidase 670 were identified as putative interactors of PrkAc. All of these 671 proteins were found phosphorylated in other organisms 672 [48,51]. Particularly, it was reported that the activity of the superoxide dismutase from *L. monocytogenes* is regulated by 673 phosphorylation at Ser and Thr residues being most active at 674 its non-phosphorylated form [70]. 675

In summary, in the present work we identify 62 candidates 676 that provide a starting point for further biochemical and cellular 677 studies. The physiological relevance of the proteins and protein 678 families identified in this interactome analysis has to be further 679 examined. According to recent proteomic meta-analysis many 680 of these proteins families (including glycolytic enzymes and 681 elongation factors) are frequently detected as differentially 682 expressed in various conditions raising concern about their 683 specificity [54,55]. Based on previous reports we can hypothesize 684 that some of these frequently identified proteins present in 685

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JOURNAL OF PROTEOMICS XX (2011) XXX-XXX



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].

692 4. Conclusions

In this work we describe for the first time a functional STPK 693 from L. monocytogenes and start to unravel the processes 694 controlled by protein phosphorylation in this human patho-695 gen. We demonstrated that PrkA is an active STPK able to 696 phosphorylate the exogenous substrate MBP at Ser and/or Thr 697 residues and able to autophosphorylate specific Thr residues 698 within its activation loop sequence. Moreover, using an 699 interactomic approach we identified 62 proteins as potential 700 interaction partners of PrkAc. The diversity of proteins identi-701 fied suggests that the signal transduction pathways mediated by 702703 this STPK in L. monocytogenes may affect a large variety of 704 fundamental biological functions including protein synthesis, cell wall metabolism, and carbohydrates metabolism. Interest-705 ingly, these processes are also regulated by phosphorylation in 706 other bacteria, suggesting that these enzymes could be control-707 ling conserved functions in prokaryotes [13,15,16,51]. 708

In addition some of the proteins identified in this study arise 709 as possible physiologically relevant interactors of PrkA. In 710 particular evidence coming from other organisms suggests 711 712 that the enzyme UDP-N-acetylglucosamine pyrophosphorylase (GlmU) implicated in peptidoglycan biosynthesis might be 713 important in PrkA signal transduction pathways. STPKs with 714 extracellular PASTA domains have been reported to bind 715 peptidoglycan fragments and to participate in the regulation 716 of cell wall synthesis and cell division in several bacteria [31]. In 717 718addition, phosphorylated residues have been identified in GlmU homologs by phosphoproteomic studies, suggesting that this 719 activity is controlled by the action of STPKs [52]. Interestingly 720 enough, the kinase reported to phosphorylate GlmU in 721

M. tuberculosis is PknB, an enzyme highly homologous to PrkA 722 [68]. 723

Also it is worth mentioning the identification of the RNA 724 binding protein Sun as an interactor of PrkA. The specific 725 recovery of this protein, which is expressed at low levels (not 726 identified previously in 2D gels of total protein extracts from 727 *L. monocytogenes*), and co-localized with this kinase in the same 728 operon points to the biological relevance of this interaction. 729 Further work is now being undertaken to validate and 730 characterize these interaction partners of PrkAc and its 731 possible biological relevance. 732

The present work provided us useful information regarding 733 selected pathways that may be regulated by kinase activity. 734 This framework will be the starting point for a more detailed 735 and comprehensive analysis of the role of this STPK in 736 bacterial physiopathology. 737

Supplementary materials related to this article can be 738 found online at doi:10.1016/j.jprot.2011.03.005. 739

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