Goals and Challenges in Bacterial Phosphoproteomics

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Received: 11 October 2019; Accepted: 12 November 2019; Published: 13 November 2019

Abstract: Reversible protein phosphorylation at serine, threonine and tyrosine is a well-known dynamic post-translational modification with stunning regulatory and signalling functions in eukaryotes. Shotgun phosphoproteomic analyses revealed that this post-translational modification is dramatically lower in bacteria than in eukaryotes. However, Ser/Thr/Tyr phosphorylation is present in all analysed bacteria (24 eubacteria and 1 archaea). It affects central processes, such as primary and secondary metabolism development, sporulation, pathogenicity, virulence or antibiotic resistance. Twenty-nine phosphoprotein orthologues were systematically identified in bacteria: ribosomal proteins, enzymes from glycolysis and gluconeogenesis, elongation factors, cell division proteins, RNA polymerases, ATP synthases and enzymes from the citrate cycle. While Ser/Thr/Tyr phosphorylation exists in bacteria, there is a consensus that histidine phosphorylation is the most abundant protein phosphorylation in prokaryotes. Unfortunately, histidine shotgun phosphoproteomics is not possible due to the reduced phosphohistidine half-life under the acidic pH conditions used in standard LC-MS/MS analysis. However, considering the fast and continuous advances in LC-MS/MS-based phosphoproteomic methodologies, it is expected that further innovations will allow for the study of His phosphoproteomes and a better coverage of bacterial phosphoproteomes. The characterisation of the biological role of bacterial Ser/Thr/Tyr and His phosphorylations might revolutionise our understanding of prokaryotic physiology.

Keywords: bacteria; Ser/Thr/Tyr phosphorylation; His phosphorylation; phosphoproteomics; differentiation; sporulation; LC-MS/MS

1. Introduction

Phosphoproteomics involves the analysis of a complete set of phosphorylation sites present in a cell. It has undergone a revolution since 2000, thanks to the advances in mass spectrometry (MS) based phosphoproteome methodologies. Large datasets describing the phosphoproteomes of several organisms were created. While nine amino acids (Ser, Thr, Tyr, His, Lys, Arg, Asp, Glu and Cys) can be modified by four types of phosphate protein linkages, only the phosphorylations at Ser, Thr and Tyr have been extensively characterised and associated with stunning regulatory and signalling cellular functions, especially in eukaryotes [1]. For instance, the human phosphoproteome harbours more than 30,000 Ser/Thr/Tyr phosphorylation sites [2]. Bacterial proteins can also be phosphorylated at Ser/Thr/Tyr, but to a much lesser extent. To date, 38 Ser/Thr/Tyr phosphoproteomic studies on bacteria have been reported, describing the phosphoproteome of 24 species of eubacteria and one species of archaea (Halobacterium salinarum) (Table 1). Mycobacterium tuberculosis harbours the largest bacterial phosphoproteome described as consisting of 500 Ser/Thr/Tyr phosphorylation sites.
sites from 301 proteins [3]. Bacterial Ser and Thr phosphorylation (average abundances of 59% and 34.1% for Ser and Thr, respectively) is much more abundant than Tyr phosphorylation (average abundance of 9.9%) (Table 1). While Ser/Thr/Tyr phosphorylation exists in bacteria, there is a consensus about that histidine phosphorylation is the most abundant protein phosphorylation in prokaryotes [4]. However, these residues have almost not been subjected to phosphoproteomic analyses. There have only been three studies describing bacterial histidine phosphoproteomes [5–7]. This is a consequence of the acid lability of the histidine phosphate linkage, which is not compatible with most of the proteomic liquid chromatography tandem–mass spectrometry (LC-MS/MS) protocols. Protein phosphorylation at amino acid residues, other than Ser/Thr/Tyr or His, is less abundant and has been poorly characterised. In this work, we review the state of the art and the challenges of bacterial Ser/Thr/Tyr and His phosphoproteomics.

Table 1. Bacterial Ser/Thr/Tyr phosphoproteomic studies. Abbreviations: n.r., not reported; Ch, chemoheterotrophic.

| Bacterium                  | Year | pSer (%) | pThr (%) | pTyr (%) | Reference |
|----------------------------|------|----------|----------|----------|-----------|
| Bacillus subtilis          | 2007 | 69.2     | 20.5     | 10.3     | [8]       |
| Escherichia coli (E. coli) | 2008 | 68       | 23       | 9        | [9]       |
| Lactococcus lactis        | 2008 | 46.5     | 50.6     | 2.7      | [10]      |
| Klebsiella pneumoniae     | 2009 | 31.2     | 15.1     | 25.8     | [7]       |
| Pseudomonas aeruginosaputida| 2009| 52.8     | 36.1     | 11.1     | [11]      |
| Halobacterium salinarum   | 2009 | 84       | 16       | 0        | [12]      |
| Mycobacterium tuberculosis| 2010 | 40       | 60       | 0        | [3]       |
| Streptomyces coelicolor    | 2010 | 34       | 52       | 14       | [13]      |
| Streptococcus pneumoniae  | 2010 | 47       | 44       | 9        | [14]      |
| Bacillus subtilis          | 2010 | n.r.     | n.r.     | n.r.     | [15]      |
| Neisseria meningitidis     | 2011 | n.r.     | n.r.     | n.r.     | [16]      |
| Streptomyces coelicolor    | 2011 | 46.8     | 48       | 5.2      | [17]      |
| Listeria monocytogenes     | 2011 | 93       | 43       | 7        | [18]      |
| Helicobacter pylori        | 2011 | 42.8     | 38.7     | 18.5     | [19]      |
| Clostridium acetobutylicum | 2012 | 40       | 50       | 10       | [20]      |
| Rhodopseudomonas palustris (Ch) | 2012| 63.3     | 16.1     | 19.4     | [21]      |
| Thermus thermophilus       | 2012 | 65.3     | 26       | 8.7      | [22]      |
| Thermus thermophilus       | 2013 | 57       | 36       | 7        | [23]      |
| Synechococcus sp.          | 2013 | 43.9     | 42.44    | 13.66    | [24]      |
| E. coli                   | 2013 | 75.9     | 16.7     | 7.4      | [25]      |
| Staphylococcus aureus      | 2014 | n.r.     | n.r.     | n.r.     | [26]      |
| Acinetobacter baumannii Abh12O-A2 | 2014| 71.8     | 25.2     | 3.8      | [27]      |
| Acinetobacter baumannii ATCC 17879 | 2014| 68.9     | 24.1     | 5.2      | [27]      |
| Pseudomonas aeruginosa     | 2014 | 49       | 24       | 27       | [28]      |
| Listeria monocytogenes     | 2014 | 64       | 31       | 5        | [29]      |
| Saccharopolyspora erythraea| 2014| 47       | 45       | 8        | [30]      |
| Bacillus subtilis          | 2014 | 74.6     | 18.6     | 7.3      | [31]      |
| Chlamydia caviae           | 2015 | n.r.     | n.r.     | n.r.     | [32]      |
| Sinorhizobium melloti      | 2015 | 63       | 28       | 5        | [33]      |
| E. coli                   | 2015 | n.r.     | n.r.     | n.r.     | [34]      |
| Bacillus subtilis          | 2015 | n.r.     | n.r.     | 22.6     | [35]      |
| Synechocystis sp.          | 2015 | n.r.     | n.r.     | n.r.     | [36]      |
| Acinetobacter baumannii SK17-S | 2016| 47       | 27.6     | 12.4     | [6]       |
| Acinetobacter baumannii SK17-R | 2016| 41.4     | 29.5     | 17.5     | [6]       |
| Mycobacterium smegmatis    | 2017 | 27.79    | 73.97    | 1.24     | [37]      |
| Mycobacterium tuberculosis | 2017 | 68       | 29       | 3        | [38]      |
| Microcystis aeruginosa     | 2018 | n.r.     | n.r.     | n.r.     | [39]      |
| Streptomyces coelicolor    | 2018 | 50.6     | 47.4     | 2        | [40]      |
| Zymomonas mobilis          | 2019 | 73       | 21       | 6        | [41]      |
| Streptococcus thermophilus | 2019 | 43       | 33       | 23       | [42]      |

Average 55.9 34.1 9.9
2. Bacterial Ser/Thr/Tyr Phosphoproteomics

Classical protein chemistry phosphoproteomic approaches require protein purification, peptide mapping and identification of phosphorylated peptide regions and sites by N-terminal sequence analysis. These kinds of analyses are tedious, and they could cover only one phosphoprotein or a few phosphoproteins. Chemistry approaches basically disappeared with the development of 2DE gel-based analyses, combined with MS or MS/MS analysis for protein identification. The most recent advances in LC-MS/MS make these traditional chemistry approaches obsolete. In this review, we describe the state of the art of a large bacterial dataset of Ser/Thr/Tyr phosphorylation identified using gel-based and LC-MS/MS-based methodologies.

2.1. Gel-Based Analyses

Classically, proteomics and phosphoproteomics are based on the use of 2DE gels. 2DE gel-based phosphoproteomic experiments use specific dyes [43] or antibodies [44] to identify and quantify phosphorylated protein spots. These methodologies are still useful, particularly in identifying possible isoforms of phosphorylated proteins [45]. However, due to the reduced bacterial Ser/Thr/Tyr phosphorylation, only a few reports describing bacterial Ser/Thr/Tyr phosphoproteomes by means of 2DE gel approaches have been reported. The phosphoproteomes of Neisseria meningitidis, Staphylococcus aureus and Chlamydia caviae were characterised by means of 2DE gel approaches [16,26,32] (Table 2).

Table 2. 2DE gel-based bacterial phosphoproteome studies.

| Bacterium                        | Year | Phosphoproteins | Phosphorylation Sites | Phosphoproteome                  | Reference |
|----------------------------------|------|-----------------|-----------------------|----------------------------------|-----------|
| Neisseria meningitidis           | 2011 | 51              | n.r.                  | Many biological processes        | [16]      |
| Staphylococcus aureus            | 2014 | 103             | 76                    | Pathogenicity and virulence       | [26]      |
| Chlamydia caviae (elementary body)| 2015 | 42              | n.r.                  | Virulence                         | [32]      |
| Chlamydia caviae (reticulate body)| 2015 | 34              | n.r.                  | Virulence                         | [32]      |

2.2. LC-MS/MS-Based Phosphoproteomic Analyses

Most phosphopeptide enrichment protocols use immobilised metal affinity chromatography (IMAC), which consists positively charged metal ions, such as Fe (3+), Ga (3+), Al (3+), Zr (4+) and Ti(4+) [46]. The most widespread method is the use of TiO₂ affinity chromatography [46]. TiO₂ affinity chromatography-based phosphoproteomics is mainly optimised for eukaryotic samples. Further work on optimising this method to study the relatively low Ser/Thr/Tyr phosphorylation present in bacteria will contribute to deepen the characterisation of bacterial phosphoproteomes. In this sense, an interesting phosphopeptide pre-enrichment method, which largely enhances TiO₂ efficiency, is the use of calcium phosphate precipitation (CPP) [47]. CPP consists of coprecipitated phosphorylated tryptic peptides with calcium phosphate at high pH levels [47]. CPP-pre-enriched samples are used for IMAC, enhancing the amount of purified phosphopeptides, which are further identified by LC-MS/MS analysis [47]. CPP has been successfully used in several eukaryotes, including humans [48,49], mice [50], plants [47] and yeasts [51]. CPP phosphopeptide pre-enrichment is also used in bacterial phosphoproteomics [17,40]. In Streptomyces coelicolor, CPP pre-enrichment increases TiO₂ LC-MS/MS-based phosphopeptide identification by five times [17].

2.2.1. Bacterial Ser/Thr/Tyr Nonquantitative LC-MS/MS-Based Phosphoproteomic Analyses

Due to low levels of Ser/Thr/Tyr bacterial phosphorylation, most bacterial Ser/Thr/Tyr phosphoproteomic studies used large amounts of protein (milligrams), obtained during the vegetative growth phase, to detect a relatively low number of phosphopeptides [17]. The aim of these studies
was to identify as many phosphosites as possible, and they do not provide information about the dynamic of this phosphorylation during bacterial development. Twenty-four phosphoproteomes from 18 eubacterial species and an Archaeon (*Halobacterium salinarum*) were analysed using nonquantitative LC-MS/MS-based phosphoproteomic approaches (Table 3). These studies, together with the 2DE gel-based studies described above, were pioneering in the characterisation of the existence of Ser/Thr/Tyr phosphorylation in bacteria, rather than in the characterisation of the variation of phosphoproteomes during their development or in response to different stimuli.

Table 3. LC-MS/MS-based nonquantitative bacterial phosphoproteome studies. Abbreviation: Ph, photoheterotrophic.

| Bacterium                | Year | Phosphoproteins | Phosphorylation Sites | Phosphoproteome                        | Reference |
|--------------------------|------|-----------------|-----------------------|----------------------------------------|-----------|
| *Bacillus subtilis*      | 2007 | 78              | 78                    | Carbohydrate metabolism                | [8]       |
| *E. coli*                | 2008 | 79              | 81                    | Similar to *Bacillus*                   | [9]       |
| *Lactococcus lactis*     | 2008 | 63              | 79                    | Over-representation of phosphothreonines| [10]      |
| *Klebsiella pneumoniae*  | 2009 | 81              | 93                    | Capsular biosynthesis                  | [7]       |
| *Pseudomonas aeruginosa* | 2009 | 39              | 61                    | Motility, transport and pathogenicity   | [11]      |
| *Pseudomonas putida*     | 2009 | 59              | 55                    | Several biochemical pathways           | [11]      |
| *Halobacterium salinarum*| 2009 | 26              | 31                    | Phosphoproteome in Archaea             | [12]      |
| *Mycobacterium tuberculosis* | 2010 | 301             | 500                   | Several biochemical pathways           | [3]       |
| *Streptomyces coelicolor*| 2010 | 40              | 46                    | Housekeeping proteins                  | [13]      |
| *Synechococcus*          | 2013 | 245             | 410                   | Two-component signalling pathway and photosynthesis | [24]      |
| *Listeria monocytogenes* | 2011 | 112             | 143                   | Virulence, translation, carbohydrate metabolism and stress response | [18]      |
| *Helicobacter pylori*    | 2011 | 67              | 126                   | Virulence                              | [19]      |
| *Clostridium acetobutylicum* | 2012 | 61              | 107                   | Carbon metabolism                      | [20]      |
| *Rhodopseudomonas palustris* | 2012 | 54              | 63                    | Carbon metabolism                      | [21]      |
| *Rhodopseudomonas palustris* | 2012 | 42              | 59                    | Carbon metabolism                      | [21]      |
| *Thermus thermophilus*   | 2012 | 48              | 46                    | Wide variety of cellular processes      | [22]      |
| *Thermus thermophilus*   | 2013 | 53              | 67                    | Central metabolic pathways and protein/cell envelope biosynthesis | [23]      |
| *Acinetobacter baumannii Abh120-A2* | 2014 | 70              | 80                    | Pathogenicity and drug resistance       | [27]      |
| *Acinetobacter baumannii ATCC 17879* | 2014 | 41              | 48                    | Several biochemical pathways            | [27]      |
| *Pseudomonas aeruginosa* | 2014 | 28              | 59                    | Extracellular virulence factors         | [28]      |
| *Sinorhizobium meliloti*  | 2015 | 77              | 96                    | Rhizobial adaptation                    | [33]      |
| *Microcystis aeruginosa* (nontoxic) | 2018 | 37              | n.r.                  | Several biochemical pathways            | [39]      |
| *Microcystis aeruginosa* (toxic) | 2018 | 18              | n.r.                  | Regulation of toxin generation          | [39]      |
2.2.2. Bacterial Ser/Thr/Tyr LC-MS/MS-Based Quantitative Phosphoproteomic Analyses

Once the existence of bacterial Ser/Thr/Tyr phosphorylation was demonstrated, the next issue to be explored was whether the bacterial phosphorylation changed during bacterial differentiation and/or in response to different developmental conditions. As stated above, phosphorylation in bacteria is dramatically lower than that in eukaryotes, making bacterial phosphoproteomics challenging, especially quantitative phosphoproteomics (i.e., analyses of the amount of specific phosphorylation sites and how they vary during development). To our knowledge, there are 15 reported quantitative phosphoproteomic studies on bacteria [6,15,17,25,29,31,34–38,40–42] (Table 4).

| Bacterium | Year | Phosphoproteins | Phosphorylation Sites | Phosphoproteome | Method | Reference |
|-----------|------|----------------|-----------------------|-----------------|--------|----------|
| Bacillus subtilis | 2010 | 27 | 45 | Phosphoproteome changes in different media | SILAC | [15] |
| Streptomyces coelicolor | 2011 | 127 | 289 | Sporulation factors, transcriptional regulators, protein kinases and other regulatory proteins | Label-free | [17] |
| E. coli | 2013 | 133 | 108 | Stationary phase | SILAC | [25] |
| Bacillus subtilis | 2014 | 141 | 177 | Stationary phase | SILAC | [31] |
| Listeria monocytogenes | 2014 | 191 | 242 | Purine biosynthesis regulated by PrfA phosphorylation | SILAC | [29] |
| Saccharopolyspora erythraea | 2014 | 88 | 109 | Carbon metabolism, environmental stress and protein synthesis affected by phosphorylation | SRM | [30] |
| E. coli | 2015 | 71 | n.r. | Phosphorylation varied during development | SRM | [34] |
| Bacillus subtilis | 2015 | 124 | 155 | Spore-specific determinants | Label-free | [35] |
| Synechococcus sp. | 2015 | 188 | 262 | Increased phosphorylation during nitrogen limitation | Dimethyl | [36] |
| Acinetobacter baumannii SK17-S | 2016 | 248 | 410 | Antibiotic resistance | Label-free | [6] |
| Acinetobacter baumannii SK17-R | 2016 | 211 | 285 | Antibiotic resistance | Label-free | [6] |
| Mycobacterium smegmatis | 2017 | 154 | 224 | Transmembrane proteins | Label-free | [37] |
| Mycobacterium tuberculosis | 2017 | 257 | 512 | Virulence | Tandem mass tag (TMT) | [38] |
| Streptomyces coelicolor | 2018 | 48 | 85 | Regulatory proteins | TMT | [40] |
| Zymomonas mobilis | 2019 | 125 | 177 | N2 fixing regulated by phosphorylation | Label-free | [41] |
| Streptococcus thermophilus | 2019 | 106 | 161 | Divisome proteins phosphorylated by the PknB kinase | Dimethyl | [42] |

The first bacterial quantitative phosphoproteomic study was performed in 2010 on Bacillus subtilis (B. subtilis) using the stable isotope labelling of amino acids (SILAC) in a cell culture, describing the changes in the B. subtilis phosphoproteome in different media [15]. In 2014, another SILAC
analysis was also performed on *B. subtilis*, analysing different developmental stages [31]. Other quantitative phosphoproteomic analyses using SILAC were performed on *Escherichia coli* (*E. coli*) and *Listeria monocytogenes* [25,29].

In 2011, we performed the first quantitative phosphoproteomic study describing the differences in a bacterium during development [17]. We used CPP combined with TiO$_2$ chromatography and LC-MS/MS to analyse *Streptomyces coelicolor* (*S. coelicolor*) Ser/Thr/Tyr phosphorylation [17]. This methodology was successful in identifying a relatively large amount of phosphorylation (127 phosphoproteins and 289 phosphorylation sites) from a relatively low protein amount (0.3 mg) [17]. Later, in 2018, our group improved the *S. coelicolor* quantitative phosphoproteome analysis by applying tandem mass tag (TMT) isobaric labelling to the protein extracts, prior to CPP/TiO$_2$ phosphopeptide enrichment and LC-MS/MS analysis [40]. Protein and phosphoprotein abundance quantification was highly improved [40]. However, phosphopeptide identification was reduced to 48 phosphoproteins [40], while 127 phosphoproteins were identified in our previous label-free analysis [17]. The lower efficiency in phosphopeptide identification in the TMT analysis [17] was probably a consequence of mixing the very low phosphorylated vegetative samples with the more highly phosphorylated reproductive stages. TMT isobaric labelling was later used to quantify the phosphoproteome variation in virulent and nonvirulent *Mycobacterium tuberculosis* strains [38]. Dimethyl labelling was also used to analyse the variation of bacterial phosphoproteomes in *Synechocystis* sp. and *Streptococcus thermophilus* [36,42].

Label-free quantitative phosphoproteomic analyses were also performed in *Bacillus subtilis*, *Acinetobacter baumannii*, *Mycobacterium smegmatis* and *Zymomonas mobilis* [6,35,37,41]. Scheduled multiple reaction monitoring (sMRM), another label-free approach that consists in selecting the masses of the ions to be sequenced in the MS/MS, was used to analyse the *E. coli* and *Saccharopolyspora erythraea* phosphoprotoomes [30,34].

3. Bacterial Proteins and Pathways Modulated by Ser/Thr/Tyr Phosphorylation

3.1. Bacterial Proteins Identified as Phosphorylated

Bacterial cellular processes including proteins identified as phosphorylated comprise carbon/protein/nucleotide metabolism, transcription, translation, protein/cell envelope biosynthesis, two-component signalling pathways, stress response, transport or extracellular proteins (Tables 2–4). These results suggest a role of Ser/Thr/Tyr phosphorylation in the regulation of central metabolism. Proteins participating in nonessential but clinically and industrially relevant cellular activities were also identified as phosphorylated. The phosphoproteomes of the pathogenic bacteria, *Staphylococcus aureus* [26], *Chlamidia caviae* [32], *Klebsiella pneumoniae* [7], *Streptococcus pneumoniae* [14], *Helicobacter pylori* [19], *Acinetobacter baumanii* [6,27] and *Mycobacterium tuberculosis* [38], include proteins related to pathogenicity and virulence as they are capsule biosynthetic proteins, proteins involved in drug resistance or proteins related to motility. *Streptomyces*, the most important source for bioactive secondary metabolites in nature (mainly antibiotics, but also antitumourals, immunosuppressors, etc.) [52], harbours Ser/Thr/Tyr-phosphorylated proteins that are involved in secondary metabolism regulation, suggesting a role of Ser/Thr/Tyr-modulating antibiotic production [17,40].

Bacteria are the most diverse group of living beings on the planet. Consequently, finding and comparing protein orthologues is not always possible. However, when we compared the 38 bacterial phosphoproteomes already known (Tables 2–4), we were able to identify 29 phosphoprotein orthologues present in at least four phosphoproteomes. These 29 phosphoproteins include 12 ribosomal proteins, four enzymes from glycolysis and gluconeogenesis, three elongation factors, two cell division proteins, one RNA polymerase subunit, one ATP synthase subunit and one enzyme from the citrate cycle (Figure 1a). Consequently, Ser/Thr/Tyr phosphorylation might modulate transcription, translation, stress response, central metabolism (glycolysis, gluconeogenesis and citrate cycle), energy production (oxidative phosphorylation) and cell division. Interestingly, the most commonly identified phosphorylated bacterial protein is the GroEL chaperone, showing 63 phosphorylation sites in
20 phosphoproteomes (Figure 1b) from 17 bacterial species. This corresponds to an average of three phosphorylation sites per protein.

Figure 1. The phosphoprotein orthologues systematically identified in the 38 bacterial phosphoproteomic studies are shown in Tables 1–4. (a) Phosphoproteins (highlighted in red) are classified by function. The bacteria, in which they were identified, are indicated. (b) Total number of the phosphorylation sites identified for each phosphoprotein orthologue in the 38 analysed bacteria, as well as the number of bacteria, in which a phosphoprotein was detected. Asterisks indicate multiphosphorylated proteins, i.e., these showing more phosphorylation sites than bacteria.

3.2. Bacterial Ser/Thr/Tyr Phosphorylation Motifs

The relatively low number of bacterial Ser/Thr/Tyr phosphorylations makes it difficult to find statistically significant phosphorylation motifs. To our knowledge, only four phosphoproteomic works reported phosphorylation motifs in bacteria: the motifs XXααααTX(X/V)ϕ(P/R)I (α is an acidic residue, and ϕ is a large hydrophobic residue) [3], and EXpT, PpT and pTXp [38] were found in Mycobacterium tuberculosis; PFxFA[T/S]GY was described in Sinorhizobium meliloti [33]; and X(pT)xEx was identified in Streptomyces coelicolor [17]. It is clear that new workflows need to be explored to identify
bacterial Ser/Thr/Tyr phosphorylation motifs. It may be interesting to combine all of the bacterial phosphorylated orthologue sequences, perhaps separated into different taxonomic groups, in the same motif search. In addition, the search algorithms might be modified to mine phosphorylation motifs in the reduced bacterial phosphoproteomes. Until significant bacterial Ser/Thr/Tyr phosphorylation motifs are found, it will be difficult to create robust bioinformatics tools to perform reliable in silico bacterial phosphorylation predictions.

3.3. Bacterial Processes Demonstrated to be Modulated by Ser/Thr/Tyr Phosphorylation

While Ser/Thr/Tyr phosphorylation is present in all of the analysed bacteria (Tables 1–3; Figure 1), there are very few bacterial processes that have been demonstrated to be regulated by Ser/Thr/Tyr phosphorylation. Some of the best characterised bacterial activities modulated by Ser/Thr/Tyr phosphorylation are cell wall metabolism, transcription and protein synthesis. Ser/Thr/Tyr kinases are required to modulate the activity of Bacillus cell wall hydrolases, in response to peptidoglycan fragments during spore germination [53] and the vegetative stage [54]. The Ser and Thr phosphorylation of Deinococcus radiodurans FtsA and FtsZ cell division proteins affect their functional interactions [55]. DivIVA, the key protein controlling apical growth in the mycelial bacterium Streptomyces, is modulated by the Ser/Thr kinase AfsK [56]. The Streptococcus suis DivIVA orthologue was also demonstrated to be modulated by a Ser/Thr kinase [57].

Other important bacterial processes were demonstrated to be regulated by Ser/Thr/Tyr phosphorylation. Quorum sensing was described to be regulated by phosphorylation in the marine pathogen, Vibrio alginolyticus [58,59]. Streptococcus suis growth is modulated by phosphorylation [60]. Gene expression was reported to be modulated by Ser/Thr/Tyr phosphorylation in Staphylococcus aureus [61] and Streptococcus [62]. The Tu elongation factor is modulated by Thr phosphorylation in Mycobacterium tuberculosis [63]. Photosynthesis was demonstrated to be modulated by Ser/Thr/Tyr phosphorylation in the model cyanobacterium, Synechocystis sp. [64]. The phosphorylation of the β subunits of phycocyanins affects the energy transfer and the state transition of Synechocystis photosynthesis [64]. Bacterial virulence can also be modulated by phosphorylation. Phosphorylation of the AmpC β-lactamase reduces β-lactamase activity and increases antibiotic resistance in Acinetobacter baumannii [6]. Xanthomonas citri virulence is activated by the phosphorylation of the Lon protease, which stabilises HrpG, the master regulator of type III secretion systems in this pathogenic bacterium [65].

4. Bacterial Histidine Protein Phosphorylation

Histidine phosphorylation was first demonstrated in bacterial two-component systems in 1980 [66]. Since then, several descriptions of two-component system signalling in prokaryotes have been reported. Histidine kinases are the most abundant protein kinases in bacteria. For instance, Streptomyces coelicolor, a bacterium harbouring the largest amount of eukaryotic-type Ser/Thr/Tyr kinases [17], has 47 Ser/Thr/Tyr kinases and 149 histidine kinases. By contrast, to the best of our knowledge, only a single His kinase in eukaryotic cells, which is highly conserved in eukaryotes and implicated in suppressing tumour metastasis, has been characterised [4,67].

4.1. Methodological Challenges

The histidine phosphate linkage has a half-life of about 30 min at pH 3 [68], which makes histidine phosphorylation incompatible with most LC-MS/MS analyses. Consequently, the characterisation of histidine phosphoproteomes remains a difficult challenge. To the best of our knowledge, there are only three reports describing His phosphoproteomic analyses on bacteria by means of standard shotgun phosphoproteomics, i.e., using acidic solvents [5–7]. Lai et al. [5] analysed the histidine phosphoproteome of nine bacteria, identifying seven and 31 phosphopeptides per bacterium [5]. They identified some pathogenicity proteins that were phosphorylated at histidine in Acinetobacter baumannii, Klebsiella pneumoniae, Vibrio vulnificus and Helicobacter pylori [5]. Lin et al. [7] and Lai et al. [6]
analysed histidine phosphorylation together with the phosphorylation of Ser, Thr, Tyr and Asp in *Klebsiella pneumoniae* and *Acinetobacter baumanii*, respectively. They found that 12.9% (in *Klebsiella*) and 4.9% (in *Acinetobacter*) of the identified phosphorylations correspond to pHis. Given that acid buffers were used in these three works [5–7], there are probably many phosphohistidines that were not identified. These works give rise to the interesting question of whether the raw data of the other Ser/Thr/Tyr bacterial phosphoproteomic works can be processed, setting pHis as a post-translational modification, to identify novel histidine phosphorylations.

Histidine phosphorylation has constituted a methodological challenge for decades. The recent development of 1- and 3-pHis monoclonal antibodies [69,70] has contributed, at least in part, to overcoming this important methodological drawback. Kleinnijenhuis et al. [71] proposed to develop a fast LC method or nonacidic solvent systems to protect phosphohistidines from acidic dephosphorylation. Reverse-phase chromatography at a neutral or basic pH value, combined with the use of negative-ion-mode MS, might also be an alternative.

4.2. Bacterial Pathways Modulated by Histidine Phosphorylation

Bacterial two-component systems are the most important form of bacterial signal transduction. Canonical two-component systems are formed by a sensor histidine kinase, usually a transmembrane receptor, and a response regulator, usually a transcriptional regulator. The sensor histidine kinase transfers the phosphoryl group to the response regulator modulating its activity. Bacterial genomes harbour huge amounts of two-component signalling systems. For instance, *Streptomyces coelicolor* harbours more than 100 two-component signalling systems (www.sanger.ac.uk), many of which regulate secondary metabolism and antibiotic production [72]. Bacterial two-component systems modulate important cellular processes, such as photoreception [73], quorum sensing [74], temperature sensing [75] and plant-bacteria interactions [76].

Histidine kinases belonging to two-component systems can be predicted in silico, since the kinases and their response regulator genes are usually located adjacently in a genome. Once identified, their putative response regulators and functions can be studied. However, there are bacterial histidine kinases beyond two-component systems, of which the biological function is much more difficult to characterise. These latter types of kinases, which are not associated with the known response regulators, also show important regulatory activities, such as chemotaxis [77] or nucleoside metabolism [75].

5. Conclusions

The huge advances in LC-MS/MS methodologies and phosphopeptide enrichment, developed over the last 20 years, has made the study of large datasets of Ser/Thr/Tyr phosphopeptides possible, mainly in eukaryotes, but also in bacteria. Ser/Thr/Tyr protein phosphorylation in bacteria is dramatically lower than that in eukaryotes. However, this important post-translational modification is present in all the analysed bacteria (Tables 1–4) and affects important cellular processes. While Ser/Thr/Tyr phosphorylation exists and is important in bacteria, there is a consensus that histidine phosphorylation is the most abundant protein phosphorylation in bacteria. However, histidine phosphoproteomes remain elusive due to the reduced phosphohistidine half-life under the acidic pH levels used in the shotgun phosphoproteomic procedures. Considering the fast and continuous advance in LC-MS/MS-based phosphoproteomic methodologies, it is expected that further innovations, such as the recent EasPhos platform developed by Humphrey et al. [78] and the development of workflows compatible with histidine phosphorylation stability, will allow for a better coverage of bacterial Ser/Thr/Tyr and His phosphoproteomes. Applying these kinds of methodologies to analyse bacterial phosphoproteomes might revolutionise our understanding of bacterial physiology.

**Author Contributions:** P.Y. and N.G.-Q. wrote the main body of the revision; G.F.-G. and S.A.-F. inspected and confirmed the bibliography; A.M. planned, revised and structured the manuscript.

**Funding:** Our group is funded by Ministerio de Ciencia, Innovación Universidades/Agencia Estatal de Investigación/Fondo Europeo de Desarrollo Regional (RTI2018-093978-B-I00), Consejería de Empleo, Industria y
Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| LC-MS/MS     | liquid chromatography tandem mass spectrometry |
| IMAC         | immobilised metal affinity chromatography |
| CPP          | calcium phosphate precipitation |
| SILAC        | stable isotope labelling by amino acids in cell culture |
| TMT          | tandem mass tag |
| sMRM         | scheduled multiple reaction monitoring |

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