Quantitative phosphoproteome analysis of *Streptomyces coelicolor* by immobilized Zirconium (IV) affinity chromatography and mass spectrometry reveals novel regulated protein phosphorylation sites and sequence motifs

Running title: The *Streptomyces* and *Escherichia* phosphoproteomes

Key words: *Streptomyces*; *Escherichia*; phosphoproteomics; Zirconium(IV)-IMAC; LC-MS/MS

Abbreviations: CPP, calcium phosphate precipitation; IMAC, immobilized metal affinity chromatography; SDC, sodium deoxycholate; TCEP, Tris (2-carboxyethyl) phosphine hydrochloride; CAA, chloroacetamide

Abstract

Streptomycetes are multicellular gram-positive bacteria that produce many bioactive compounds, including antibiotics, antitumorals and immunosuppressors. The Streptomyces phosphoproteome remains largely uncharted even though protein phosphorylation at Ser/Thr/Tyr is known to modulate morphological differentiation and specialized metabolic processes. We here expand the S. coelicolor phosphoproteome by optimized immobilized zirconium (IV) affinity chromatography and mass spectrometry to identify phosphoproteins at the vegetative and sporulating stages. We mapped 361 phosphorylation sites (41% pSer, 56.2% pThr, 2.8% pTyr) and discovered four novel Thr phosphorylation motifs ("Kxxxx(pT)xxxxK", "DxE(pT)", "D(pT)" and "Exxxxx(pT)") in 351 phosphopeptides derived from 187 phosphoproteins. We identified 154 novel phosphoproteins, thereby almost doubling the number of experimentally verified Streptomyces phosphoproteins. Novel phosphoproteins included cell division proteins (FtsK, CrgA) and specialised metabolism regulators (ArgR, AfsR, CutR and HrcA) that were differentially phosphorylated in the vegetative and in the antibiotic producing sporulating stages. Phosphoproteins involved in primary metabolism included 27 novel ribosomal proteins that were phosphorylated during the vegetative stage. Phosphorylation of these proteins likely participate in the intricate and incompletely understood regulation of Streptomyces development and secondary metabolism. We conclude that Zr(IV)-IMAC is an efficient and sensitive method to study protein phosphorylation and regulation in bacteria and enhance our understanding of bacterial signalling.

1. Introduction

Streptomycetes are important industrial bacteria producing two thirds of the bioactive secondary metabolites used in clinical settings, mainly antibiotics, but also antitumourals and immunosuppressors [1]. Streptomycetes exhibit complex morphological differentiation in which substrate vegetative mycelium differentiate into sporulating aerial mycelium and this process is accompanied by the production of generally bio-active or specialized metabolites [2]. The nature of the metabolic changes accompanying this differentiation process is poorly understood in streptomycetes [3, 4]. Reversible protein phosphorylation is likely to play role in this process [5-8], however, molecular details of such regulatory processes remains to be characterised.

Published phosphoproteomic studies of *S. coelicolor* revealed 184 proteins *O*-phosphorylated at Ser/Thr/Tyr residues [5-8]. Many of these proteins were differentially phosphorylated during *Streptomyces* development [6, 7], including several key regulatory proteins [9]. However, to the best of our knowledge, the biological relevance of *Streptomyces* Ser/Thr/Tyr phosphorylation was experimentally validated for only seven proteins: FtsZ, the first component of the bacterial divisome, whose phosphorylation pleiotropically affects actinorhodin and undecylprodigiosin production [7]; DivIVA, a protein that controls polar growth and hyphal branching [10]; DnaA, a protein controlling DNA replication [11]; MreC and Pbp2 that participate in peptidoglycan synthesis [12]; AfsR, a transcriptional activator of secondary metabolism [13]; and AfsK, a Ser/Thr kinase that globally controls the biosynthesis of several secondary metabolites [14]. AfsR is phosphorylated by AfsK [15]. The importance of these proteins is demonstrated by the fact that *dnaA* and *divIVA* knockouts are not viable [16, 17]. Whereas the *Streptomyces ftsZ*, *afsR*, *afsK, mreC* and *pbp2* mutants are

viable, they show a dramatic alteration of development [18], hyphae branching [10], spore wall thickness [12] and secondary metabolism [15, 19].

The activity of FtsZ was reported to be modulated by S/T/Y phosphorylation in other bacteria, such as *Deinococcus radiodurans* [20] and *Mycobacterium tuberculosis* [21]. DivIVA activity was also described to be modulated by phosphorylation in *Streptococcus suis* [22]. In addition to cell division, other important cellular processes were reported to be modulated by S/T/Y phosphorylation in bacteria as cell-wall remodelling, quorum sensing or bacterial virulence (reviewed in Yagüe et al, 2019 [9]). Clearly, further studies are required to fully characterise and understand the *Streptomyces* phosphoproteome and other bacterial phosphoproteomes [9].

Published *Streptomyces* phosphoproteome studies displayed very little overlap between the reported sets of protein phosphorylation sites [5-8], indicating that the *Streptomyces* phosphoproteome is complex and dynamic and that additional molecular features of phosphorylation-mediated signalling remains to be discovered in this species. We hypothesized that our recently reported sensitive and robust Zr(IV)-IMAC method for phosphopeptide enrichment [23] could expand the experimentally verified *Streptomyces* phosphoproteome, and other bacterial phosphoproteomes. Here, we optimised and automated the Zr(IV)-IMAC protocol for analysis of *E. coli* and *S. coelicolor* bacterial phosphoproteomes. We then applied Zr(IV)-IMAC and mass spectrometry (LC-MS/MS) to compare the phosphoproteomes of the *S. coelicolor* vegetative mycelium and sporulating aerial mycelium. We observed novel regulatory features of protein phosphorylation and greatly expanded the number of known phosphorylation sites and phosphoproteins in this important industrial bacterium.

2. Material and methods

2.1. Experimental design

E. coli cultures were analysed at the stationary stage as reported before[24]. *S. coelicolor* was analysed at two key developmental stages: vegetative substrate mycelium hypha (30 hours) and aerial mycelium sporulating hyphae (65 hours). The 65 hour culture comprises a mixture of old vegetative mycelium and young sporulating aerial hyphae. Quantitative phosphoproteomics was performed by processing and analysing three biological replicates of each developmental stage. The identified *E. coli* and *S. coelicolor* phosphoproteomes identified in this work were compared with previously reported phosphoproteomes (Table 1, Fig. 1d-e and Fig. S1c-e).

2.2. Bacterial strains and media

S. coelicolor M145 [25] and *E. coli* W3110 were used in this study. *Streptomyces* was grown in Petri dishes (85 mm diameter) with 25 mL of solid GYM medium (5 g/L glucose, 4 g/L yeast extract, 5 g/L malt extract, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5g/L K_2HPO_4 added after autoclaving, 20 g/L agar) [26] that were covered with cellophane disks, inoculated with 100 µL of a spore suspension (1 x 10^8 viable spores/mL), and incubated at 30 ºC. *Streptomyces* cells were collected at 30- (substrate mycelium) and 65-hours (aerial sporulating mycelium) using a plain spatula.

E. coli cultures were grown as described before [24] on liquid M9 minimal medium, consisting of M9 salts (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl) supplemented with 0.5% (w/v) glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, with vigorous shaking at 37 °C. Cells were collected by centrifugation at stationary phase (OD600 = 1.0) and washed three times with ice cold PBS.

2.3. Protein extraction and digestion

Protein extracts from *E. coli* and *S. coelicolor* were digested as previously reported [24] [27] with some alterations. Cells were washed three times with cold PBS before the pellets were resuspended in 8mL lysis buffer containing 8M Urea (Acros Organics), 100mM Tris-HCl pH 8.5 (Acros Organics), 1.5% sodium deoxycholate (SDC, Sigma-Aldrich), 5mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma-Aldrich), 30 mM chloroacetamide (CAA, Acros Organics), 10 U/ml DNaseI (Invitrogen), 1 mM sodium orthovanadate (Sigma-Aldrich), complete mini EDTA free (Roche) + phosphoSTOP (Roche) and then ruptured with a probe sonicator (MSE Soniprep 150) in an ice-bath for 45 min in cycles of 20 sec power and 40 sec break (total time approx. 2h 20min). The cell debris was removed by centrifugation at 18,000g for 1h at 4ºC (rotor F15-6 x 100y (Thermo Scientific) and the supernatant was diluted three times using 16mL of dilution buffer (100mM Tris + 5mM TCEP + 30 mM CAA + 1 mM magnesium chloride; Acros Organics), also containing proteases and phosphatase inhibitors, before adding benzonase (Merck Millipore) to 1% and incubating for 2h at room temperature.

Proteins were precipitated by chloroform/methanol precipitation. One part of sample was mixed with 4 parts methanol (VWR), 1-part chloroform (Acros Organics) and 3 parts mQH_2O with thorough vortexing after each addition (216 mL total in five tubes: four with 45 mL and one with 36 mL). Tubes were centrifuged for 10 min at 5,000 rpm at room temperature, then the upper layer (H_2O/methanol mixture) was discarded. The bottom layer (chloroform) was mixed with 3 parts methanol and then sonicated and centrifuged again. The solvent was removed, and the pellet was left to air dry.

The protein pellet was re-dissolved in digestion buffer (100 mM Tris-HCl pH 8.5 + 1% SDC + 5 mM TCEP + 30 mM CAA) and protein concentration was estimated using Pierce™ reducing agent compatible microplate BCA protein assay kit (Thermo Scientific). Proteins were digested overnight at room temperature with 5% w:w Trypsin (Promega) and 1% w:w Lys-C (Wako). After digestion, SDC was precipitated by acidifying to pH 3.5 with 10% formic acid (FA, Sigma-Aldrich) and centrifuging at 14,000 rpm for 10 min at 4°C. The supernatant was then loaded on a tC18 Sep-Pak cartridge (Waters) that was previously conditioned with cold 95% acetonitrile (MeCN, Sigma-Aldrich) and then equilibrated with cold 0.1% FA. The column was washed two times with cold 0.1% FA and peptides were eluted with cold 30% MeCN. A small aliquot of 25 µL from the elution fraction was taken before lyophilizing. The dry aliquot was re-dissolved in 25 µL water and peptide concentration was estimated using a NanoPhotometer® N60/N50 (Implen) with preprogramed protein UV with QC Ratio (260/280).

2.4. Calcium phosphate precipitation (CPP) of phosphopeptides

The protocol for CPP was performed as previously reported [28]. A total of four solutions were prepared: solution 1 (0.5M Na_2PO_4 (Sigma-Aldrich), solution 2 (2M NH_4OH), solution 3 (2M $CaCl_2$ (Sigma-Aldrich) and wash solution (80mM $CaCl_2$). For each replicate, 200 µg of peptides were dissolved to 400 µL with mQH_2O and the pH was adjusted to 9-10 using solution 2, then added 32 µL of solution 1 and solution 2 and mixed. 32 µL of precipitation solution 3 were added and the mixture was vortexed for 1 min before centrifugation at 20,000g for 10 min at room temperature. The supernatant was discarded (containing non-phosphorylated peptides) and 480 µL of wash solution were added to the pellet, mixing up and down with the pipette until suspended, then

centrifuged again at 20,000g for 5 min. The washing and centrifugation steps were repeated once more, and the liquid was once again discarded. The pellet was dissolved in 160 µL of ice-cold 5% FA. The phosphopeptides were de-salted using tC18 Sep-Pak cartridges (Waters) as described previously.

2.5. Phosphopeptide enrichment using zirconium(IV)-IMAC HP magnetic microparticles

This protocol was adapted from Arribas Díez et al. (2021) [23] and performed using a KingFisher Duo workstation (Thermo Scientific). All solvents utilized were ice-cold. Dried peptides were re-dissolved in water to a final concentration of 4 µg/µL and diluted to a final volume of 250 µL with binding solvent (80% MeCN + 5% trifluoroacetic acid -TFA, Sigma-Aldrich- + 0.1M glycolic acid -GA, Sigma-Aldrich-). Zr-IMAC HP magnetic beads (Resyn Biosciences) were first equilibrated for 1min in 500uL binding solvent before incubation with the peptide solution for 5 min. Beads were washed three times for 2 minutes (first in binding solvent, then in wash solvent 1 (80% MeCN + 1% TFA) and last wash solvent 2 (10% MeCN + 0.2% TFA), and phosphopeptides were eluted using 1.25M NH_4OH (pH 10-11) solution. Elution fractions were lyophilized before analysis by LC-MS/MS.

2.6. Phosphopeptide analysis by LC-MS/MS

Dried phosphopeptides were re-dissolved in 2% MeCN and analysed on a Dionex Ultimate 3000 RSLCnano system (Thermo Scientific) coupled online to an ESI Orbitrap Fusion Lumos® tribrid mass spectrometer (Thermo Scientific). Injected peptides were on-line de-salted using a C18 PepMapTM 100 trap column (300µm i.d. x 5mm, 5µm, 100Å, Thermo Scientific) for 2 min using 2% MeCN + 0,1% FA. Trapped

peptides were eluted and separated on a 75µm i.d. x 18cm column packed in-house with ReproSil-Pur 120 C18 3um particles (Dr. Maisch)[29] at a flow-rate of 250 nL/min with a 90 min linear gradient of 2-34% solvent B (95% MeCN + 0.1% FA) against solvent A (100% H_20 + 0.1% FA).

The ESI mass spectrometer was set to Data-Dependent Acquisition mode. Precursor ion (MS) spectra were acquired in the range m/z 375 - 1600 (2+ to 5+ ion charge state) at a mass resolution setting of 60,000 at m/z 400 in profile mode, with an AGC target of 1e6 ions. During a cycle time of 3 secs, the most intense precursor ions were automatically selected for fragmentation with higher-energy collisional dissociation (HCD) and dynamically excluded for 15 sec. Fragments were analysed in the Orbitrap at a mass resolution setting of 30,000 at m/z 400 in centroid mode.

2.7. Database search and data analysis

Raw files were processed using the Proteome Discoverer 2.5 software tool (Thermo Scientific) and searched against protein databases (SwissProt database filtered for *S. coelicolor* taxonomy; Uniprot database *E. coli* strain K12 UP000000318) using Mascot 2.6 (Matrix Science). Precursor mass tolerance was set to 5 ppm and fragment mass tolerance was set to 0.05 Da with a limit of 2 missed tryptic cleavages. *O*-phosphorylation of serine, threonine and tyrosine, oxidation of methionine and deamidation of asparagine and glutamine were set as variable modifications, while *S*-carbamidomethylation of cysteine was set as fixed modification. Low confident peptides were excluding using a Mascot ion score threshold of ≥ 18 and a FDR of ≤ 0.01 through Percolator[30]. Phosphorylation sites were assigned using ptmRS [31] for calculating phosphorylation localization probabilities, with only phosphoRS phosphosite scores of ≥ 95% being accepted. Duplicated phosphopeptides (those

harbouring the same phosphosite, i.e. phosphopeptides with partial digestions, methionine oxidations, cysteine carbamidomethylation, glutamine deamidation) were filtered, keeping the phosphopeptide with the lowest Mascot identification p-value (percolator PEP) in *E. coli* (Table S1) and with the lowest quantification q-value in *S. coelicolor* (Table S2). Peptide scores, precursor charge, peptide m/z, ptmRS scores, for all phosphopeptides, and other details are shown in Tables S1 and S2.

Label free quantitative proteomics was performed in the *Streptomyces* substrate mycelium hyphae (30 hours) and aerial sporulating hyphae (65 hours). Samples were processed in triplicate. The significance of the phosphopeptide abundances estimated with Proteome Discoverer 2.5 were analysed using the MetaboAnalyst 5.0 software (Institute of Parasitology, McGill University, Montreal, Quebec, Canada) [32]. The MetaboAnalyst parameters were as follows: the missing values were estimated as 1/5 of the minimum positive value of each variable; the abundance values were log10-transformed and scaled using the "pareto scaling" method. The differences in phosphopeptide abundances were considered significant if the q-value was equal to or less than 0.05 and the log2 65h/30h ratio was greater than 1 or lower than -1. Differentially phosphorylated proteins were filtered and abundance-corrected by subtraction of proteins that were previously found by us to be already differentially expressed by quantitative proteomics [7]. In this way we discarded phosphopeptides originating from proteins that exhibited similar significant differences of abundance (i.e. both, phosphopeptide and protein, up-regulated or down-regulated). We retained phosphopeptides from proteins exhibiting non-significant abundance changes at 30 and 65 hours [7], as well as phosphopeptides showing opposite trends as compared to the cognant protein (i.e. phosphopeptides up-regulated, protein down-regulated or vice-versa) (Table S2). The abundances of the rest of the phosphopeptides identified were

considered non-significant (tabs b1 and b2 in Table S2): phosphopeptides that exhibited significant abundance changes from cognant proteins showing the same significant up- or down-regulation at 65 hours compared to 30 hours (i.e. proteins that changed in abundance, without a real change in the stoichiometry of phosphorylation) (tab b1 in Table S2); phosphopeptides without significant abundance changes independently of the protein abundances (tab b2 in Table S2).

Phosphopeptides showing significant changes in abundance values were clustered according to their temporal abundance profiles by applying a Z-score transformation of the protein abundances and a Euclidean distance function.

Phosphorylation motifs were searched by means of the MoMo software [33] using the motif-x algorithm, a p-value threshold of 0.001 and the *S. coelicolor* proteome as context.

2.8. Analysis of protein functions

Proteins were classified into functional categories according to their annotated functions in the Gene Bank database, publications and by homology/functions according to the Gene Ontology[34], the Conserved Domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), and the KEGG Pathway (http://www.genome.jp/kegg-bin/show_organism?org=sco) databases.

2.9. Analysis of phosphopeptide hydrophobicity, acidity, basicity and length

The length of detected phosphopeptides was analyzed as was the the content of hydrophobic, basic, acidic and neutral amino acid residues by Peptide 2.0 software (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php), and the

significance was analysed using Student's t-test (p value equal to or less than 0.01 (asterisks in Fig. 1f and Fig. S1f).

3. Results

3.1. Optimisation of Zirconium(IV)-IMAC based phosphoproteomics

We first tested and optimised our Zr(IV)-IMAC protocol for bacterial phosphoproteome analysis of *E. coli* (Fig. S1 and Table S1). In two previous studies [6, 7], we observed that calcium phosphate precipitation (CPP) of phosphopeptides [28] enhanced TiO_2 phosphopeptide enrichment. To test whether this was also the case for Zr(IV)-IMAC we compared Zr(IV)-IMAC phosphopeptide enrichment with and without prior CPP treatment. Surprisingly, CPP reduced the number of identified phosphopeptides from *E. coli* (Fig. S1a). We then tested different peptide loading amounts (200 and 800 µg) and different Zr(IV)-IMAC bead/peptide ratios (1:2 and 2:1). The most efficient workflow, in terms of number of identified phosphopeptides and the phosphopeptide selectivity was using 200 µg protein digest and a 2:1 Zr(IV)-IMAC bead/peptide ratio (Fig. S1a,b). For *E. coli* protein samples, we identified 340 phosphorylation sites (71.2% pSer, 20.6% pThr, 8.2% pTyr) in 339 phosphopeptides derived from 223 phosphoproteins by using Zr(IV)-IMAC and LC-MS/MS (Table S1).

We then applied the Zr(IV)-IMAC (2:1 bead/peptide ratio) phosphopeptide enrichment protocol to *S. coelicolor* with and without prior CPP treatment. As observed for *E. coli* samples, CPP reduced zirconium(IV)-IMAC efficiency for phosphopeptide recovery from *Streptomyces* samples (Fig. 1a). We concluded that the CPP method was unnecessary when using Zr(IV)-IMAC for phosphopeptide enrichment in *E. coli* and *S. coelicolor*.

3.2. Quantitative phosphoproteomics by Zirconium(IV)-IMAC for studies of Streptomyces coelicolor development

We studied the role of protein phosphorylation in relation to activation of *Streptomyces* secondary metabolism. We performed Zr(IV)-IMAC and label-free quantitative mass spectrometry analysis of two *Streptomyces coelicolor* developmental stages: substrate/vegetative mycelium (30h, non-producer of antibiotics) and sporulating aerial mycelium (65 hours) [35]. These are the developmental stages at which S/T/Y phosphorylation was reported to be the most abundant [6, 7]. Three biological replicates were processed and analysed by Zr(IV)-IMAC and LC-MS/MS to sequence, identify and quantify phosphopeptides (outlined in Fig. 1b).

We identified 361 phosphorylation sites (41% pS, 56.2% pT, 2.8% pY) in 351 phosphopeptides derived from 186 *S. coelicolor* phosphoproteins (Fig. 1c) (Table S2). Forty percent (40%) of the phosphorylation sites and phosphopeptides were more abundant in the 30-hours samples than in the 65-hour samples. About 20% of the phosphorylation sites exhibited the opposite behaviour, whereas the extent of phosphorylation was at a similar level at both time points in approximately 40% of the cases (Fig. 1c) (Table S2).

Previous *S. coelicolor* phosphoproteomic studies using TiO_2 affinity enrichment and LC-MS/MS [5-8] reported 184 *Streptomyces* phosphoproteins with little overlap to the sets of proteins identified in the present study (Table 1) (Fig. 1d). Here, we identified 154 novel phosphoproteins, representing a vast expansion of the phosphoproteome. Thus a total of 338 phosphoproteins are now experimentally identified in *S. coelicolor* (Fig. 1e) (Table S2).

3.3. Analysis of phosphorylation sites

We identified 361 phosphorylation sites in *S. coelicolor* showing a distribution of 41% pS, 56.2% pT and 2.8% pY, which is in accordance with previous *Streptomyces* phosphoproteome reports (Table 1). Prediction of putative phosphorylation site sequence motifs using the MoMo software [33] identified four putative pT motifs: "Kxxxx(pT)xxxxK", present in 4 phosphopeptides; "DxE(pT)", present in 7 phosphopeptides; "D(pT)" present in 22 phosphopeptides; and "Exxxxx(pT)" present in 17 phosphopeptides (Fig. 2).

The STRING database [36] allowed the identification of putative functional interaction clusters between most of the proteins harbouring the putative phosphorylation motifs identified (Fig. 2), which seems to indicate that Thr phosphorylation might be regulating distinctive cellular processes: protein synthesis (FusA) and folding (GroEL1 and GroEL2) in the case of "Kxxxx(pT)xxxxK"; protein synthesis (RpsD), DNA synthesis (TopA topoisomerase) and oxidative stress (SodF1) in the case of "DxE(pT)"; protein synthesis (RpsO), protein folding (DnaN, DnaK), amino acid synthesis (AlaS, CarB) and protein phosphorylation (PkaB) in the case of "DxE(pT)"; protein folding (DnaK, GroEL1), peptidoglycan (DapF) and membrane (YidC) synthesis in the case of "Exxxxx(pT)".

Notably, more than 50% (28 phosphopeptides) of the 50 phosphopeptides harbouring the putative pT phosphorylation motifs were differentially phosphorylated in the substrate and in the aerial mycelium. Two proteins, PkaB and DasR, were increasingly phosphorylated at the aerial mycelium stage (65h) whereas the 26 remaining proteins were increasingly phosphorylated at the substrate mycelium stage (30h) (Table S2).

3.4. Differences in the substrate and aerial mycelium phosphoproteomes

Comparative analysis of the two developmental stages analysed (substrate and aerial mycelium) revealed that the abundance (scaled by the "pareto scaling" method) of 181 phosphopeptides showed significant variations between the 30- and 65-hour cultures. A majority of 127 phosphopeptides showed low degrees of phosphorylation at 65-hours whereas 54 phosphopeptides exhibited extensive phosphorylation at 65-hours (Fig. 3a, Table S2).

The 181 phosphopeptides showing significant abundance variations were clustered according to their temporal abundance profiles applying a Z-score transformation of the protein abundances and a Euclidean distance function. This approach generated three main clusters of peptide abundances (Fig. 3b) (Table S2). Phosphopeptides from primary metabolism proteins were mostly present in clusters 1 and 2 (highly phosphorylated at 30-hours), whereas regulatory proteins were mostly present in cluster 3 (highly phosphorylated at 65 hours). The heat maps showed in Fig. 3b also illustrate the very good reproducibility between the three biological replicates analysed at each developmental time point.

Figure 4 shows the abundances of the 181 phosphopeptides from 109 phosphoproteins displaying significant abundance variations: 100 phosphopeptides obtained from 71 primary metabolism proteins (DNA replication/transcription, nucleotide metabolism, glycolysis and glyconeogenesis, pentose phosphate pathway, aerobic energy production, amino acid metabolism, protein synthesis and folding, RNA/protein processing, nucleases); 32 phosphopeptides from 15 regulatory proteins including the transcriptional regulators ArgR, AfsR, CutR, HrcA and DasR [4, 37-40] and the kinases PkaA/B, AfsK [13, 14]; 25 phosphopeptides from 7 stress and defence proteins (chaperones, superoxide dismutases, catalases, phage restriction systems); 6 phosphopeptides from the protein translocase SecA, the membrane protein insertase

YidC and the potassium transporter ATPase SCO3717; and 13 phosphopeptides from 9 septation and cell division proteins (DnaA, MurG, CrgA, FtsK, SepF2) [41-44] (Fig. 4) (Table S2). Five phosphopeptides from 4 proteins with unknown function were also detected (not shown in Fig. 4) (Table S2).

Primary metabolism, stress and defence proteins as well as SecA, YidC and SCO3717 were mostly phosphorylated at 30h (substrate mycelium) whereas transcriptional regulators and kinases, were mostly phosphorylated at the aerial mycelium stage (65-hours) (Fig. 4). Septation /cell division proteins were differentially phosphorylated in the substrate vegetative and aerial sporulating mycelium. These results suggest a role of S/T/Y phosphorylation in the regulation of primary and secondary metabolism as well as in the regulation of vegetative and sporulation cell divisions.

There are 128 phosphorylation sites in 128 phosphopeptides derived from 88 phosphoproteins showing non-significant variations in their level of phosphorylation between 30- and 65-hours (Table S2). These include 82 phosphopeptides from primary metabolism proteins (mainly ribosomal proteins but also nucleotide and amino acid biosynthetic enzymes); 6 phosphopeptides from the transporter SCO5084 present in the actinorhodin biosynthetic cluster; 15 phosphopeptides from stress and defence proteins (mainly chaperones); 12 phosphopeptides from regulatory proteins (transcriptional regulators kinases); and 7 phosphopeptides from cell division proteins (StrE1 sortase, HU DNA binding protein, SepF3) (Table S2).

The abundance of 45 phosphorylation sites in 42 phosphopeptides from 38 phosphoproteins could not be accurately quantified due to their low abundance (Table S2). These include 24 phosphopeptides from primary metabolism proteins (mainly biosynthetic enzymes); 1 phophopeptide from SCO5087, a biosynthetic protein present

in the actinorhodin biosynthetic cluster; 4 phosphopeptides from the protein secretory apparatus (SecA, SecE, SecF); 7 phosphopeptides from stress and defence proteins (chaperones); 4 phosphopeptides from kinases and transcriptional regulators; 1 phosphopeptide from the FtsK DNA translocase; and 1 phosphopeptide from the putative peptidase SCO0575 with unknown function (Table S2).

3.5. S/T phosphorylation sites are located at discrete structural protein regions

We next analysed the phosphorylation sites of proteins whose function was experimentally demonstrated to be modulated by S/T phosphorylation, namely DnaA and AfsK [14, 45], as well as proteins whose structure and functional domains are known, DasR and FtsK [44, 46]. Fig. 5 shows the Alphafold predicted structures [47] of these proteins with annotation of the phosphorylation sites identified in this study.

The chromosomal DNA replication DnaA protein was demonstrated to be phosphorylated at T486 [11]. This phosphorylation alters the mutual arrangement of domains III (ATP binding) and IV (DNA binding) and affects DnaA activity [11, 45]. We identified phosphorylations at S108 and T294, both located at the beginning of the long non-globular domain II, with a flexible linker that interacts with domain I [45] (Fig. 5a). This phosphorylation might modulate the protein interactions and flexibility of the DnaA II domain (Fig. 5a).

The *S. coelicolor* AfsK Ser/Thr kinase was described to be auto-activated by autophosphorylation at T168 [14]. In this study we identified additional 6 AfsK phosphorylations, including T170 and S163, close to T168 that might enhance T168 auto-activation (Fig. 5b).

The crystal structure of the *S. coelicolor* DasR global transcriptional regulator was recently elucidated [46]. DasR is formed by two globular domains, the DNA-

binding domain (which interacts with DNA) and the effector-binding domain (which interacts with sugars) [46]. At the N-term, DasR forms a linear tail of amino acids whose function remains uncharacterised. The conformation of this region was also not elucidated in the crystal DasR structure (PDB accession number PDB-ID 4ZS8) [46] and the confidence of the N-term tail Alphafold prediction for this region was very low (orange colour in Fig.5c), indicating that it might correspond to a disordered region that may change conformation upon phosphorylation. We demonstrated that this N-term tail was phosphorylated at three threonine residues (Fig. 5c). The biological function of these phosphorylations remains unknown, but they might alter DasR conformation and modulate the function of the DNA- and/or the effector -binding domains. Two of the DasR phosphorylation sites belonged to the "D(pT)" and "Exxxxx(pT)" putative phosphorylation motifs identified in this work (Fig. 2).

The *S. coelicolor* FtsK cell division protein (involved in DNA translocation) harbours a winged-helix-turn-helix (wHTH) DNA interaction domain (amino acids 849-915) [44]. We identified two FtsK phosphorylations at S305 and T407, located far from the wHTH domain (Fig. 5c) that probably do not modify the binding ability of the FtsK to DNA, but might modulate the FtsK DNA translocator activity.

4. Discussion

The study of bacterial S/T/Y phosphoproteomes presents a major challenge because the stoichiometry of protein phosphorylation is very low in bacteria as compared to eukaryotes [9]. Most reported bacterial phosphoproteome studies used relatively large amounts of protein (>1 milligram) and TiO_2 based phosphopeptide enrichment methods to identify relatively few phosphopeptides [9], usually on the order of 50-150 phosphorylation sites (Table 1). Fe(III)-IMAC was recently used in studies of

E. coli, to identify novel phosphoproteins [24]. In the present study, we report for the first time on zirconium(IV)-IMAC as a phosphopeptide enrichment method for *Escherichia* and *Streptomyces*. We optimised the zirconium(IV)-IMAC phosphopeptide enrichment protocol for *E. coli*, identifying 340 phosphorylation sites in 339 phosphopeptides from 223 phosphoproteins, including 9 novel phosphoproteins (Table S1). We then applied the Zirconium(IV)-IMAC and LC-MS/MS workflow to analyse and compare the phosphoproteomes of the vegetative (30 hours) and sporulating (65 hours) stages of *S. coelicolor*. We identified 361 phosphorylation sites in 351 phosphopeptides derived from 186 phosphoproteins. We identified 154 novel phosphoproteins, almost doubling the number of experimentally detected *S. coelicolor* phosphoproteins. Thr phosphorylation sites are highly prevalent in *Streptomyces* compared to the *E. coli* (50% vs. 20% respectively, Table 1), but consistent with those reported in other *Streptomyces* phosphoproteome studies (Table 1). Interestingly, 50 of the 178 pT phosphorylation sites identified here fell inside four putative phosphorylated sequence motifs (Fig. 2), whereas no phosphorylation motifs could be found for the 130 pS sites and the 9 pY sites. Most of the proteins harbouring pT motifs belong to primary metabolism (protein synthesis and folding, DNA synthesis, oxidative stress, amino acid synthesis, peptidoglycan and membrane synthesis) and they are more extensively phosphorylated in the substrate mycelium (30-hour culture) than in the aerial mycelium. In contrast, other proteins harbouring pT motifs such as the PkaB and AfsK kinases, well-known regulators of secondary metabolism [13], and the global transcriptional regulator DasR, that is also modulating secondary metabolism [40], are increasingly phosphorylated in the 65-hour culture (aerial mycelium stage) than in the 30h culture. These results suggest the existence of a relatively homogeneous group of Thr targeting kinases controlling the phosphorylation at the identified pT motifs and modulating

primary and secondary metabolism. Further work is required to identify these kinases and enhance our understanding of the regulation of *Streptomyces* secondary metabolism.

The overlap between our Zr(IV)-IMAC based study and previous TiO_2 based phosphoproteomic analyses was low for *E. coli* (20.7%) [48-50] (Fig. S1c), even lower for *Streptomyces coelicolor* (9.5%) [5-8] (Fig. 1e), and much lower than that observed for similar studies of eukaryotic samples, i.e. 44% overlap between Zr(IV)-IMAC and TiO_2 [23]. This might be related to the low level of S/T/Y phosphorylation in bacteria as compared to eukaryotes [9], which makes phosphopeptide enrichment more challenging. In fact, Zr(IV)-IMAC phosphopeptide enrichment selectivity was low in *E. coli* (<12%) (Fig. S1b) and in *S. coelicolor* (18.1%), which is in contrast with eukaryotic samples where phosphopeptide enrichment selectivity is often beyond 90% [23]. Interestingly, 208 of the *E. coli* phosphoproteins identified here by Zr(IV)-IMAC and LC-MS/MS (92.4% of the total) were identified in recent studies using Fe(III)-IMAC [24, 51] (Fig. S1d), indicating that Zr(IV)- and Fe(III)-IMAC have similar phosphopeptide affinity, as already reported for eukaryotes [23]. Clearly, the chosen phosphopeptide enrichment method (TiO_2 or Zr(IV)-IMAC / Fe(III)-IMAC) and protocols influence the outcome of the study. Interestingly, phosphopeptides indentified only by Zr(IV)-IMAC phosphopeptide enrichment were shorter and had more neutral amino acids than those identified only by TiO_2, in both, *S. coelicolor* and *E. coli* (Fig. 1f and Fig. S1f). Until recently, TiO_2 was the phosphopeptide enrichment method used in most bacterial phosphoproteome studies (Table 1) [9]. Obviously, re-analyzing the bacterial phosphoproteomes using alternative phosphopeptide enrichment methods such as Zr(IV)-IMAC will further enhance our knowledge of bacterial phosphoproteomes.

Mapping the experimentally determined phosphorylation sites to computationally predicted 3D structures of several *S. coelicolor* proteins show that they locate to discrete structural and functional domains [14, 44-46, 52, 53] (Fig. 5). In addition, many of the phosphorylation sites identified are located in regions with low Alphafold predictive score [47], possibly corresponding to intrinsically disordered regions that may change conformation upon phosphorylation (Fig. 5). Thus, the identified S/T/Y phosphorylation sites might modulate the conformations, interactions and activities of important *Streptomyces coelicolor* regulatory proteins.

Fig. 6 summarises key proteins that were differentially phosphorylated in 30 and 65-hour *S. coelicolor* cultures (vegetative and aerial mycelium stages, respectively). Most of these proteins were not known to be phosphorylated (highlighted in red). Primary metabolism proteins, mainly ribosomal proteins and anabolic enzymes, were mostly phosphorylated in the vegetative mycelium (30-hour cultures), whereas key secondary metabolism regulators (CutR, HrcA, DasR) [39, 40, 54] were phosphorylated at the aerial mycelium stage (65-hours) (Fig. 4). Consequently, both, primary and secondary metabolism, seems to be modulated by S/T/Y phosphorylation. Phosphorylation of ribosomal proteins was revealed to influence subunit association and to enhance translation rate in *Streptomyces coelicolor* [55]. Therefore, the ribosomal protein phosphorylation detected here might modulate protein rate synthesis during the vegetative and reproductive stages. One of the clearest morphological differences between substrate and aerial mycelium hyphae, is the low branching of the aerial hyphae [56], that might be influenced by the phosphorylation of the AfsK S/T kinase, a key regulator of hyphae branching [10] that is differentially phosphorylated at 30- and 65-hours (Figs. 4 and 6). Vegetative and sporulation cell divisions are complex and differentially regulated [57]. Key cell division proteins such as SepF2, a paralog of

SepF (involved in the Z-ring membrane anchoring [58]), and the FtsK/CrgA sporulation proteins [43, 59], are differentially phosphorylated in vegetative and aerial mycelium indicating that protein phosphorylation is involved in the regulation of *Streptomyces* cell division.

The functions of the novel protein phosphorylation sites identified in this work, as well as those of the majority of previously reported phosphoproteins in *S. coelicolor*, remains to be characterized. As mentioned above, the activity of only seven *Streptomyces* proteins was demonstrated to be directed modulated by S/T/Y phosphorylation: DivIVA and AfsK, both controlling polar growth and hyphal branching [10]; AfsR controlling secondary metabolism [13]; DnaA, controlling DNA replication [11]; MreC and Pbp2 controlling peptidoglycan synthesis [12]. Further work will be necessary to fully understand the biological role of *Streptomyces* S/T/Y phosphorylation, including mutagenesis of the phosphorylation sites mimicking phosphorylation/non-phosphorylation, analysis of S/T/Y kinase mutants, comparison of the 3D structure of the phosphorylated/unphosphorylated proteins, pull-down experiments, etc. Nevertheless, the *S. coelicolor* phosphoproteome contributes to enhance our appreciation of the potential molecular regulatory features of *Streptomyces*.

In conclusion, Zirconium(IV)-IMAC is a sensitive and robust phosphopeptide enrichment method to study bacterial phosphoproteomes. This methodology allowed us to identify novel phosphoproteins in *Streptomyces coelicolor*, almost doubling the size of the verified phosphoproteome of this bacterium. The general application of zirconium(IV)-IMAC in combination with mass spectrometry will further extend our knowledge of bacterial phosphoproteomes.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [PubMed ID: 34723319] partner repository with the dataset identifier PXD031551.

Tables

Table 1

Overview of the S/T/Y phosphoproteins identified in phosphoproteomics of *Streptomyces coelicolor* and *Escherichia coli*. The phosphopeptide enrichment methodology used is indicated.

	Phosphopeptide enrichment method	pS/pT/pY (%)	Protein starting amount (µg)	Phosphoproteins	Nº phosphoproteins / µg protein	Reference
Escherichia coli	TiO_2	68 / 23 / 9	20,000	78	0.004	[48]
	TiO_2	75.9 / 16.7 / 7.4	9,800	135	0.01	[49]
	TiO_2	59.6 / 33.5 / 3.9	50	409	8.18	[50]
	Fe(III)-IMAC	64.7 / 26.7 / 8.6	2,000	781	0.4	[24]
	Fe(III)-IMAC	62.9 / 22.5 / 14.6	Not reported	384	--	[51]
	Zr(IV)-IMAC	71.2 / 20.6 / 8.2	184.6	223	1.2	This work
Streptomyces coelicolor	TiO_2	34 / 52 / 14	50,000	40	0.0008	[5]
	TiO_2	46.8 / 48 / 5.2	300	127	0.42	[6]
	TiO_2	48 / 52 / 0	100	48	0.5	[7]
	TiO_2	51.4 / 41.7 / 6.9	Not reported	52	--	[8]
	Zr(IV)-IMAC	41 / 56.2 / 2.8	553.8	189	0.3	This work

Figures

Fig. 1. Characterisation of Phosphoproteomics of *Streptomyces coelicolor* by Zr(IV)-IMAC and mass spectrometry.

(a) Number of phosphopeptides identified using Zr(IV)-IMAC with and without CPP pre-enrichment. (b) Quantitative phosphoproteomic workflow. (c) Number of *S. coelicolor* phosphorylation sites, phosphopeptides and phosphoproteins identified. (d) Number of *S. coelicolor* phosphoproteins identified using TiO_2 based phosphopeptide enrichment phosphoproteomic studies [5-8]. (e) Number of *S. coelicolor* phosphoproteins identified using Zr(IV)-IMAC (this work) and TiO_2 [5-8] based phosphopeptide enrichments. (f) Box and whisker plots illustrating the percentage of hydrophobic, basic, acid and neutral amino acids in the 154 and 152 phosphoproteins identified by TiO_2 or Zr(IV)-IMAC phosphopeptide enrichment methods; phosphopepitide length is also shown. Asterisks indicate significant differences (p-value less than 0.01).

Fig. 2. Phosphothreonine motifs and putative protein interaction clusters. Motif-x frequency logos and putative STRING functional interaction protein clusters are shown.

(a) "Kxxxx(pT)xxxxK" motif, 4 phosphopeptides. (b) "DxE(pT)" motif, 7 phosphopeptides. (c) "D(pT)" motif, 22 phosphopeptides. (d) "Exxxxx(pT)", 17 phosphopeptides.

Fig. 3. Quantitative phosphopeptide abundances in 30- and 65-hour grown cultures.

(a) Volcano plot of the abundances and q-values of the phosphopeptides identified. Significant (q-value less than 0.05, log2 65h/30h ratio greater than 1 or lower than -1; protein and phosphopeptide abundances significantly different) more (red) and less

(blue) abundant phosphopeptides are indicated. (b) Heat-maps showing the abundance of phosphopeptides presenting significant differences. The number of phosphopeptides belonging to different functional categories into each abundance cluster are shown. The most abundant functional categories are highlighted in red.

Fig. 4. Phosphopeptide abundances grouped by function.

Relative abundances (log2 65h/30h) for phosphopeptides from proteins involved in primary metabolism, stress and defence, transport, septation/cell division and regulation (transcriptional regulators, kinases) are shown. All abundances of protein and phosphopeptide shown are significantly different (q-value less than 0.05, log2 65h/30h ratio greater than 1 or lower than -1) between the 30 and the 65-hour samples. The SCO numbers of key proteins discussed in the manuscript are indicated. Dashed lines indicate the 2-fold abundance threshold. Novel phosphoproteins not identified in previous works are highlighted in bold font.

Fig. 5. Predicted 3D structures of selected *S. coelicolor* proteins and their experimentally determined phosphorylation sites.

Structure prediction by AlphaFold. The phosphorylation sites identified in this study are labelled. DnaA, DasR and FtsK functional domains are indicated.

Fig. 6. *S. coelicolor* integrated phosphoproteome variations during development. Substrate (30 hours) and sporulating aerial mycelium (65 hours). Key proteins discussed in the text are shown at the developmental stage in which they display significant up-regulation. The number of phosphopeptides identified for each protein is indicated

between brackets. Novel phosphoproteins not identified in previous works are highlighted in red.

Supplementary data

Fig. S1. *Escherichia coli* Zr(IV)-IMAC phosphoproteomics.

(a) Number of phosphopeptides identified using Zr(IV)-IMAC with and without CPP pre-enrichment and using different proportions Zr(IV)-IMAC bead/peptide ratios. (b) Zr(IV)-IMAC phosphopeptide selectivity using different amounts of phosphopeptides. (c-e) Comparison of the *E. coli* phosphoproteins identified using Zr(IV)-IMAC (this work), TiO_2 [48-50] and Fe(III)-IMAC [24, 51]. (f) Box and whisker plots illustrating the percentage of hydrophobic, basic, acid and neutral amino acids in the 380 and 98 phosphoproteins identified by TiO2 or Zr(IV)-IMAC phosphopeptide enrichment methods; phosphopepitide length is also shown. Asterisks indicate significant differences (p-value less than 0.01).

Table S1

Escherichia coli phosphopeptides. Novel phosphoproteins not identified in previous works are highlighted in red.

Table S2

Streptomyces coelicolor phosphopeptides. Novel phosphoproteins not identified in previous works are highlighted in red. (a) Phosphopeptides showing significant abundances. (b) Phosphopeptides showing no-significant abundances (q-value greater than 0.05, log2 fold change between +/- 1). (c) Phosphopeptides no quantified.

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