Phosphoproteomics in Microbiology: Protocols for studying *Streptomyces coelicolor* differentiation

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Summary

The extension and biological role of Ser/Thr/Tyr phosphorylation in prokaryotes has been only scarcely studied. In this chapter, we describe the state of the art of microbial phosphoproteomics, focusing on protocols used for studying the phosphoproteome of *Streptomyces coelicolor*, one of the bacteria encoding the largest number of eukaryote-like Ser/Thr/Tyr kinases.

1. Introduction

Reversible protein phosphorylation at serine, threonine, and tyrosine residues is a well-known dynamic post-translational modification with stunning regulatory and signaling potential in eukaryotes *[1]*. In contrast, the extension and biological function of Ser/Thr/Tyr protein phosphorylation in bacteria are in most cases only poorly defined. Histidine/aspartate phosphorylations are common in bacterial two-component systems *[2,3]*, but its extension and biological role remains basically unknown. This is a direct consequence of the high instability of histidine phosphorylation in the acidic conditions used in phosphoproteomic protocols *[4]*. This chapter focus on bacterial Ser/Thr/Tyr phosphorylation.

During the last years, large-scale Ser/Thr/Tyr phosphoproteome studies were reported for *E. coli [5,6]*, *Streptococcus pneumonia [7]*, *Klebsiella pneumoniae [8]*, *Lactococcus lactis [9]*, *Pseudomonas [10]*, *Bacillus subtilis [11]*, *Halobacterium salinarum [12]*, *Clostridium acetobutylicum [13]*, *Streptomyces coelicolor [14,15]*, *Mycobacterium tuberculosis [16]*, *Acinetobacter baumanii [17]*, *Listeria monocytogenes [18]*, *Rhodopseudomomas palustris [19]*, *Thermus termophilus [20*],

Helicobacter pylori [21], or *Staphylococcus aureus [22]* (Table 1). The extent of protein phosphorylation in bacteria is dramatically low in comparison to eukaryotes, making bacterial phosphoproteomics a challenge. Most bacterial phosphoproteomic studies only analyzed the vegetative stages, due to the fact that differentiation is uncharacterized for most bacteria. The only two studies analyzing different developmental stages in bacteria, demonstrated that phosphorylation is dramatically higher at the differentiated stages of *S. coelicolor [15]* and *E. coli [6]*. Quantitative phosphoproteomics is challenging, and to our knowledge, there are only two published gel-free quantitative phosphoproteomic studies in bacteria: the label-free quantitative phosphoproteomics performed in *S. coelicolor [15]*, and the stable isotope labeling by amino acids in cell culture (SILAC) performed in *E. coli [6]*. All published mass spectrometry based bacterial phosphoproteomics studies applied TiO_2 for phosphopeptide enrichment, because the low phosphorylation levels of prokaryotes made other methods, such as Immobilized Metal Affinity Chromatography (IMAC), inadequate. Most of the bacterial phosphoproteomic studies used rather large amounts of protein starting material (milligrams) obtained during the vegetative growth state to detect relatively low numbers of phosphopeptides (Table 1).

In this chapter we describe the mass spectrometry based experimental workflow and analytical methods used for studying the *Streptomyces coelicolor* phosphoproteome *[15]*, which consists of pre-fractionation of peptide samples by means of calcium phosphate precipitation (CPP) of phosphopeptides *[23]* prior to titanium dioxide (TiO_2) affinity-enrichment of phosphopeptides. CPP enhances dramatically the number of Ser/Thr/Tyr phosphopeptides identified.

Streptomycetes produce most of the biologically active compounds used in biomedicine *[24-27]*. They have complex life cycles (reviewed in *[28]*), and represent the evolutionary origin of several protein domains that are known to regulate eukaryotic signalling pathways, including eukaryotic-like protein kinases *[29]*. *Streptomyces coelicolor*, the model *Streptomyces* strain *[30]*, encodes 47 predicted eukaryote-like protein kinases, twice the number of kinases predicted from genomes of other well characterized bacteria, including *E. coli* and *Bacillus subtilis* (Table 1), and it has been demonstrated to be a good model for the study of bacterial Ser/Thr/Tyr phosphorylation *[15]*.

2. Materials

2.1. *Streptomyces* strain and developmental conditions

1. GYM medium *[31]*: 5 g/l glucose, 4 g/l yeast extract, 5 g/l malt extract, 0.5 g/l $MgSO_4.7H_2O$, 20 g/l agar. Before plating, supplement with 0.5 g/l K_2HPO_4 (add 2ml per liter of a stock solution of 0.25g/ml).

2. Cellophane disks: Use cellophane from bookshop. Cut the cellophane into discs of the diameter of a petri plate, before autoclaving.

3. Incubator (30ºC).

2.2. Protein extraction and sample preparation

1. Lysis buffer: 2% SDS, 50 mM Tris-HCl pH 7, 150 mM NaCl, 10 mM $MgCl_2$, 1mM EDTA, 7 mM ß-mercaptoethanol, EDTA-free Protease Inhibitor Cocktail Tablets (e.g. Roche) and 1% phosphatase inhibitor (e.g. mixtures 1 and 2 from Sigma).

2. Centrifuge
3. Vacuum centrifuge
4. Acetone, pure
5. Ethanol (EtOH), absolute
6. Washing solution 1: 50% (v/v) H_2O, 25% (v/v) EtOH, 25% (v/v) acetone
7. Distilled water
8. 12,000 Da dialysis tubing (e.g. Sigma)
9. Bradford reagent or an alternative method for protein concentration determination
10. Dithiothreitol (DTT): 0.5 M DTT stock solution
11. Ammonia in water (NH_3-H_2O): 2M NH_3 in water
12. Iodoacetamide (IAA): 150 mM IAA stock
13. Trypsin

2.3. Calcium phosphate precipitation (CPP)

1. Solution 1: 0.5M Na_2HPO_4
2. Solution 2: 2M $NH_3 \cdot H_2O$
3. Solution 3: 2M $CaCl_2$
4. Washing solution 2: 80mM $CaCl_2$
5. Formic acid: 5% (v/v) formic acid

6. Acetonitrile (ACN): 50% (v/v) ACN

7. POROS® R3 (Applied Biosystems) reversed-phase media.

2.4. Enrichment of phosphopeptides using TiO_2 and desalting

1. C8 disks (3M™ Empore™ High Performance Extraction Disk).

2. ACN

3. TiO_2 : Titansphere, 5µm (e.g. GL Sciences).

4. Loading solution: 1M glycolic acid, 5% (v/v) trifluoroacetic acid (TFA) (sequencing grade), 80% (v/v) ACN (HPLC grade).

5. Washing solution 3: 80% 8(v/v) ACN (HPLC grade), 1% (v/v) TFA (sequencing grade).

6. Ammonia in water (NH_3-H_2O): 2M NH_3 in water

7. Elution solution 1: 40% (v/v) ACN/1M NH_3-H_2O

8. Formic acid: 3.5% (v/v) formic acid

9. POROS® R3 (Applied Biosystems) reversed-phase media.

10. Equilibration solution: 0.5% (v/v) formic acid

11. Elution solution 2: 50% (v/v) ACN, 0.5% (v/v) formic acid

12. Elution solution 3: 70% (v/v) ACN, 0.5% (v/v) formic acid

2.5. LC-MS/MS analysis

1. LC-ESI-MS/MS system: For example, a nano EasyLC system (Thermo Fisher Scientific, Odense, Denmark) interfaced by an ESI source to a LTQ-Orbitrap XL or an Q-Exactive tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).
2. Formic acid: 0.5% (v/v) formic acid
3. Fused silica column: in-house packed fused silica column, 16 cm length, 100 μm inner diameter, 375 μm outer diameter, filled with ReproSil, C18 AQ 3 μm; (e.g. Dr. Maisch, Ammerbuch, Germany)
4. HPLC solvent A: 0.1% (v/v) formic acid
5. HPLC solvent B: 90% (v/v) ACN, 0.1% (v/v) formic acid
6. ProteomeDiscoverer Software version 1.2 (Thermo Scientific)
7. Mascot server version 2.3.02 (Matrix Science)

3. Methods

Experimental workflow is detailed in the next paragraphs and outlined in Figure 1.

3.1. *Streptomyces* strain and developmental conditions

Use the model *Streptomyces coelicolor* M145 strain *[30]*, or another *Streptomyces* whose genome is sequenced. *Streptomyces coelicolor* developmental cycle and culture conditions are well known *[28]*. As introduced above, one of the keys to increase the number of detected phosphorylation sites in *Streptomyces* is to analyse

different developmental stages: vegetative, MI; and reproductive, MII/sporulation *[15]*. In the case of *S. coelicolor*, solid sporulating cultures can be prepared as described by Manteca et al. *[15]*: inoculate plates of GYM medium, covered with cellophane disks, with 100 µl of a spore suspension (1 x 10^7 viable spores/ml); incubate them at 30ºC; analyse three key developmental stages (12h, MI; 24h, MII; and 72h, sporulating MII).

3.2. Protein extraction and sample preparation

1. Collect the mycelial lawns of *S. coelicolor* M145 (or alternative species) growing on cellophane disks at different time points (12, 24, and 72 hours) using a plain spatula. Process as many biological replicates as possible, ideally, those necessary to ensure rigorous statistical analysis. Multiple replicates adds to the workload, specially MS instrumentation time, and may not always be feasible. Process at least two biological replicates for each developmental stage.

2. Lyse the cells boiling the samples in lysis buffer (10 minutes). Use 65 mg of fresh weight mycelium per ml of lysis buffer (*see* **Note 1**).

3. Centrifuge at 20,000 x g for 10 minutes.

4. Collect the supernatant.

5. Reduce sample viscosity by sonication (4 cycles of 10 seconds, on ice).

6. Precipitate the protein with acetone/ethanol (1volume sample / 4 volumes ethanol / 4 volumes acetone). Store over night at -20 ºC (*see* **Note 2**).

7. Centrifuge at 20,000gs for 10 minutes at 4ºC.

8. Discard the supernatant.

9. Wash the pellet with precooled (-20ºC) washing solution 1.

10. Repeat steps 7 and 8.

11. Resuspend the pellet in precooled (4ºC) distillated water.

12. Dialyze the sample against large volumes of water (1 hour at 4 ^{o}C, change water every 15 minutes) (*see* **Note 3**).

13. Determine protein concentration (e.g. Bradford method *[28]* or any other alternative)

14. Lyophilize samples in aliquots (for instance 100 µg of protein per aliquot).

15. Store the lyophilized samples at -80 °C (*see* **Note 4**).

3.3. In solution trypsin digestion.

1. Resuspend the protein in distilled water (add 25 µl of water to 50 µg of protein) (*see* **Note 5**).

2. Add DTT to a final concentration of 5 mM (0.25 µl of the 0.5M DTT stock to 25 µl). Adjust the pH to 8 with 2M NH_3-H_2O (*see* Note 5).

3. Incubate at 37 ºC for 45 minutes.

4. Take out the sample from 37 ºC, leave it at room temperature until it cool down.

5. Add IAA to a final concentration of 15 mM (2.5 µl of the 150 mM IAA stock), check the pH (*see* **Note 6**).

6. Incubate at room temperature for 45 minutes, in the dark.

7. Add the same amount of DTT as in step 2.

8. Add trypsin (0.5 µg of trypsin to the 25 µg of protein).

9. Incubate at 37ºC over night.

3.4. Phosphopeptide enrichment – CPP pre-fractionation/TiO_2.

1. Adjust the volume of the peptide solution (50 µg) to 100 µl with distilled water.

2. Adjust the pH of the sample solution to 9-10 using 2M NH_3-H_2O (*see* **Note 6**).

3. Add 8 µl of precipitation solutions 1 and 8 µl of precipitation solutions 2 to the 100 µl sample solution and mix.

4. Add 8 µl of precipitation solution 3 and vortex.

5. Centrifuge at 20,000 x g for 10 minutes at room temperature.

6. Discard the supernatant (this supernatant contains the non-phosphorylated peptides and can be stored to compare it with the phosphopeptide-enriched sample).

7. Add 120 µl of washing solution 2 to the pellet, vortex until it is suspended.

8. Centrifuge at 20,000 x g for 5 minutes.

9. Repeat steps 6 - 8.

10. Discard the supernatant.

11. Dissolve the pellet with 40 µl of 5% (v/v) formic acid.

12. Desalt the resulting solution by reverse phase chromatography as detailed below (section 3.5); store the dried phosphopeptides at -20ºC (*see* **Notes 4 and 7**).

13. Prepare TiO_2 microcolumns putting a plug of the C8 disk into the narrow end of the tip and load the TiO_2 material on top (*see* **Note 8**).

14. Wash the column with 30 µl of 100% ACN.

15. Resuspend the dried CPP pre-enriched sample (step 12) in 25 µl of loading solution.

16. Load the sample on the TiO_2 microcolumn.

17. Collect the flow through and load it a second time on the same column.

18. Wash with 25 µl of loading solution.

19. Wash with 25 µl of washing solution 3 (two times).

20. Elute the peptides with 10 µl of 2M NH_3-H_2O (two times).

21. Elute with 2 µl elution solution 1 to elute the phosphopeptides that bind to the C8-disk.

22. Vacuum dry the sample, resuspend it in 30 µl 3.5% (v/v) formic acid (FA), and desalt it using the protocol detailed below (section 3.5).

3.5. Sample desalting

1. Prepare an oligoR3 RP column into a p200 pipette tip with C18 plug. Equilibrate the column with 50 µl equilibration solution. (*see* **Note 9**).

2. Load the acidified peptide mixture onto the column.

3. Wash the column with 50 µl equilibration solution

4. Elute peptides in a two-step elution of peptides with 30 µl elution solution 2 and 20 µl elution solution 3.

5. Combine the eluates and dry down in a vacuum centrifuge. Store at −20 °C until further analysis (*see* **Note 7**).

3.6. LC-MS/MS analysis

1. For LC-MSMS analysis resuspend samples in 0.5% (v/v) formic acid.

2. Analyse samples using nanoliter flow chromatography (nanoLC) interfaced to a high mass resolution electrospray tandem mass spectrometer (LC-ESI-MS/MS). For example a nanoliter flow EasyLC system (Thermo Fisher Scientific, Odense, Denmark) coupled to a Q-Exactive ESI tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) can be used. (*see* **Note 10**).

3. Separate peptides using an in-house packed fused silica

4. Inject 5 µl of peptide/phosphopeptide sample onto the column.

5. Separate peptides using a linear 50 min gradient from 0% to 34% of solvent B with a constant flow of 250 nl/min.

6. Record mass spectra in in the positive ion mode with data dependent acquisition. Acquire survey scans in the m/z range 400–2000 in the Orbitrap at a mass resolution of 60000 FWHM. Perform collision induced dissociation MS/MS of 5-10 most intense ions in the linear ion trap with an activation time of 15 s.

7. Convert raw data into peak lists using for example Proteome Discoverer software. Generate mgf files, perform database searches using for example Mascot server (version 2.3.02; Matrix Science), search against the NCBInr

database. Use the following parameters to filter the results: precursor mass tolerance 10 ppm, fragment ion mass tolerance of 0.8 Da, fixed modifications: carbamidomethylation of Cys residues; variable modifications: phosphorylation of Ser/Thr/Tyr, oxidation of Met; enzyme: trypsin, two missed cleavages allowed (*see* **Note 11**).

3.7. Criteria to validate candidate phosphopeptides

Candidate phosphopeptides are validated by manual inspection of their respective MS/MS spectra using the next criteria (see Manteca et al. *[15]* for examples). Peptides should have:

1. At least 4 consecutive y or b ions.

2. An intense signal should be assigned to ions produced by fragmentation at peptide bond N-terminal to proline if proline is present in the sequence.

3. Phosphoserine sites were assigned by the appearance of a 69Da (dehydroSer) and 167Da (phosphoserine, 87+80 Da) distance between fragment ions.

4. Phosphothreonine sites were assigned by the appearance of 83Da (dehydroThr) and 181Da (phosphothreonine, 80 + 101 Da) distance for phosphothreonine.

5. Phosphotyrosine containing peptides were validated by the observation of a mass increase of 80Da to unmodified peptide and the presence of the immonium ion at m/z 216 (singly charged) or a mass difference of 243Da between fragment ions in spectra.

3.8. Label free quantitative analysis

The relative abundance of the phosphopeptides at different developmental stages is estimated based in the averaged counts per second following the next criteria (see Manteca et al. *[15]* for examples):

1. Phosphopeptides were only considered for quantification if they were detected and sequenced in two biological replicates.

2. Average abundance was calculated in the 24 LC-MS/MS runs.

3. For the LC-MS/MS runs in which the precursor ion was not sequenced, it was searched manually using a tolerance of 0.14 min for retention time and 5 ppm as mass tolerance.

4. Relative abundances were considered as significant when the average counts per second +/- SD were not overlapping between developmental stages.

4. Notes

1. Collect the sample in lysis buffer in ice. Boil the samples in the SDS-lysis buffer as soon as possible to inactive phosphatases/proteases and to stabilize the phosphoproteome.
2. Ethanol/acetone precipitation is critical to remove SDS.
3. Dialysis cleans rests of SDS and salts, but it is not critical.

4. In order to minimize methodological variation, prepare single use aliquots, process them as soon as possible, and use samples with the same storage times.

5. Do not use a buffer during trypsin digestion, because it interferes with CPP. The stock of IAA (150 mM) should be prepared freshly for each digestion. Prepare it in water, and adjust the pH to 8 with 2M NH_3-H_2O (*see* also Note 1).
6. Adjust the pH to 9-10 is critical for CPP. pH should be checked adding minimal volumes (around 0.2 µl) to pH strips.
7. Take into account that CPP is a phosphopeptide pre-enrichment, and most peptides in the CPP pre-enriched sample are not phosphorylated
8. In order to improve reproducibility of the label free quantification between replicates, TiO_2 microcolumns should be as homogeneous as possible.
9. OligoR3 RP microcolumn should be big enough to retain all the peptides. This can be checked analyzing the absence of peptides in the column flow through by MALDI-TOF.
10. It is recommended to perform 2 to 5 replicate analysis.
11. Take into account that most of the peptides identified in the final CPP-TiO_2 enriched sample are not phosphorylated due to the low level of Ser/Thr/Tyr phosphorylation in bacteria.

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Table 1: Comparison of *S. coelicolor* phosphoproteome with other published prokaryotic and eukaryotic phosphoproteomes.

The amount of protein used for the phosphoproteomic experiments, the number of genome ORFs, and number of phosphoproteins/phosphopeptides/phosphosites are shown. n.r. not reported.

	Bacterium	Protein (mg)[1]	Phosphoproteins	Phosphopeptides	Phosphorylation sites	Reference
Gram +	*S. coelicolor*	0.3	127	260	289[2]	Manteca et al. (2011) [15]
	S. coelicolor	50	40	44	46	Parker et al. (2010) [14]
	B. subtilis	10	78	103	78	Macek et al. (2007) [11]
	C. acetobutylicum	2	61	82	107	Bai et al. (2012) [13]
	L. lactis	20	63	102	79	Soufi et al. (2008) [9]
	M. tuberculosis	n.r.	301	380	500	Pisic et al. (2010) [16]
	S. pneumoniae	1	84	102	163	Sun et al. (2010) [7]
	L. monocytogenes	10	112	155	143	Misra et al. (2011) [18]
	S. aureus	50	108	n.r.	76	Bäsell et al. (2014) [22]
Gram -	*E. coli*	20	79	105	81	Macek et al. (2008) [5]
	K. pneumoniae	30	81	117	93	Lin et al. (2009) [8]
	P. aeruginosa	1.2	39	57	61	Ravichandran et al. (2009) [10]
	P. putida	1.2	59	56	55	Ravichandran et al. (2009) [10]
	H. pylori		67	82	126	Ge et al. (2011) [21]
	R. palustris (Ch)	2	54	100	63	Hu et al. (2012) [19]
	R. palustris (Ph)	2	42	74	59	Hu et al. (2012) [19]
	T. thermophilus	100	48	52	46	Takahata et al. (2012)

						[20]
	E. coli	9.8	133	n.r.	108	Soares et al. (2013) [6]
	A. baumanii Abh12O-A2	9	70	n.r.	80	Soares et al. (2014) [17]
	A. baumanii ATCC 17879	9	41	n.r.	48	Soares et al. (2014) [17]
Archaea	*H. salinarum*	20	26	42	31	Aivaliotis et al. (2009) [12]
Eukarya	*H. sapiens*	6	7832	>50000	38229	Sharma et al. (2014) [32]

[1] Total amount used in the all LTQ-Orbitrap or 2D gel runs

[2] This is one example out of numerous human phosphoproteome studies.

FIGURE LEGENDS

Fig. 1. Overview of the *S. coelicolor* phosphoproteomics workflow. Mycelium from three developmental stages is lysed by boiling in SDS and sonication; proteins are precipitated with acetone-ethanol; trypsin digestion is followed by phosphoenrichment using CPP-TiO_2. Phosphopeptides are separated on nano-HPLC, mass-measured (MS) and fragmented (MS/MS) in the high performance LTQ-Orbitrap mass spectrometer.