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Association between intrinsic disorder and serine/threonine phosphorylation in
Mycobacterium tuberculosis

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Abstract

Serine/threonine phosphorylation is an important mechanism to regulate protein function. In eukaryotes phosphorylation occurs predominantly in intrinsically disordered regions of proteins. While serine/threonine phosphorylation and protein disorder are much less prevalent in prokaryotes, *M. tuberculosis* has both high serine/threonine phosphorylation and disorder. Here I show that, similar to eukaryotes, serine/threonine phosphorylation sites in *M. tuberculosis* are highly enriched in intrinsically disordered regions, indicating similarity in substrate recognition mechanism of eukaryotic and *M. tuberculosis* kinases. Serine/threonine phosphorylation has been linked to the pathogenicity and survival of *M. tuberculosis*, thus better understanding of how its kinases recognize their substrates could have important implications in understanding and controlling the biology of this deadly pathogen.

Introduction

Reversible phosphorylation of serine and threonine residues is a widespread post-translational modification in eukaryotes, with more than a third of proteins phosphorylated during their lifetime [1]. Phosphorylation can modify protein interactions, enzyme function, localization or degradation. While traditionally, regulation and signal transduction in bacteria was thought to be mediated by histidine and aspartate phosphorylation in two-component systems, recently the occurrence and importance of phosphorylation of serine/threonine (S/T) residues has gained much attention [2–4]. Large scale mass-spectrometry based analysis has revealed S/T phosphorylation in a number of bacteria [2]. *M. tuberculosis* shows the highest rates of phosphorylation among studied bacteria with 8 % of the proteins identified as phosphorylated [5].

One of the most interesting findings about serine/threonine phosphorylation in eukaryotes is its association with intrinsically disordered regions[6–11]. Intrinsically disordered regions lack a well-defined three-dimensional structure and are characterized by low content of hydrophobic amino acids and high net charge [12]. These characteristic physiochemical properties allow accurate prediction of disordered regions across proteomes [13]. Disordered regions are often associated with binding to multiple partners in a transient manner [14–19]. These regions may undergo disorder to order transition upon binding, with decrease in conformational entropy. This uncouples binding affinity and specificity, allowing highly specific interactions to be reversible [20,21]. It has been proposed that due to high surface accessibility and transient mode of interactions, disordered regions are ideally suited for regulation by reversible phosphorylation

[6,7,20,21]. Disordered regions are highly abundant in eukaryotes with about a third of the proteins predicted to have at least one long (> 30 residues) disordered region and about 19 % of the residues in disordered state [22]. In contrast, most bacteria have much less disorder in their proteome, with about 4% proteins with long disordered regions and about 6% disordered residues [22]. The association between disorder and serine/threonine phosphorylation has not been investigated in prokaryotes.

Among bacteria, *M. tuberculosis* has one of the highest disorder content, with about 10 % disordered residues [22]. Thus, *M. tuberculosis* has both high level of S/T phosphorylation and disorder content. This prompted me to ask whether the association seen between S/T phosphorylation and disorder in eukaryotes might be present in *M. tuberculosis*, which indeed was found to be the case.

Materials and Methods

Data on *M. tuberculosis* phosphoproteins and phosphosites was obtained from Prisic *et al.* [5]. This study identified 301 phosphoproteins and 500 S/T phosphorylation events. For 215 of these sites, the specific residue that was modified could be identified with high confidence. *M. tuberculosis* proteome was obtained from Tuberculist database [23]. For disorder prediction I utilized IUPred program [24]. IUPred method is based on the observation that disordered regions do not form sufficient favorable interactions to fold, and thus have high estimated energy content [25]. I also utilized ESpritz program [26], which is conceptually different from IUPred. This method is machine learning based predictor which was trained on experimentally characterized disordered regions (missing regions in X-ray structures in PDB). I also used MFDp2 disorder

prediction tool, which is an ensemble disorder prediction tool [27,28]. Secondary structure prediction was done at Network Protein Sequence Analysis (NPSA) server [29] using consensus approach [30]. To calculate statistical significance of enrichment of pS/T sites in disordered and loop regions, I also used randomization test. For example, 84 pS/T sites are observed as disordered by IUPred program out of total 215 pS/T. For testing enrichment, I randomly selected equal number of S/T sites from each phosphoprotein as observed (1000 randomizations), and counted number of randomizations in which 84 or more sites were observed in disordered regions. This was not observed in any of 1000 randomization; hence p -value is less than $1e-3$. To analyze conservation of S/T sites, seven mycobacterium genus were chosen which are least similar to *M. tuberculosis* with respect to BLAST sequence similarity score (*Mycobacterium sp. MCS*, *M. vanbaalenii*, *M. smegmatis*, *M. chubuense*, *Mycobacterium sp. JDM601*, *M. gilvum* and *M. abscessus*). Orthologs of *M. tuberculosis* in mycobacteria were identified using reciprocal best blast approach [31], and aligned using Clustal Omega [32]. Alignment positions with gaps were excluded from the analyses. Out of 215 sites, 135 sites were present in proteins with orthologs in all 7 species. Of these 135, 103 sites were without gaps using Clustal Omega. Positions with replacement of serine with threonine and vice-versa were considered as conserved. Phosphosite data for other bacteria was obtained from respective publications [33–40].

Results

Mass-spectrometry based analysis had previously revealed 301 phosphoproteins in *M. tuberculosis* with 500 S/T phosphorylation sites [5]. First, I tested whether phosphoproteins in *M. tuberculosis* are more likely to be disordered (i.e. have long (≥ 30 residues) disordered

114 regions). I utilized IUPred program to predict disordered regions at the proteome wide level [25].
115 Phosphoproteins are about twice as likely to be disordered compared to non-phosphoproteins
116 (29.6 % vs. 13.4% respectively, Fisher p 4e-12). Since longer proteins are also more likely to
117 have long disordered regions, I tested whether phosphoproteins have higher percentage of
118 disordered residues. Phosphoproteins also have higher percentage of disordered residues
119 compared to non-phosphoproteins (16.7% vs. 12.0% respectively, two tailed t-test p 3e-5).

120
121 Out of 500 phosphorylation events detected in *M. tuberculosis*, the phosphoresidue could be
122 identified for 215 sites with high confidence [5]. For these sites, I tested whether phosphorylated
123 S/T (pS/T) residues are more likely to be disordered compared to non-phosphorylated S/T
124 (npS/T) residues from the same set of proteins. Overall 39.1% of pS/T sites are disordered
125 compared to 22.4% of npS/T sites (Fisher test p 6e-8 and randomization test p < 1e-3, Figure 1).
126 The results are very similar when another disorder prediction method, Espiritz[26] was used
127 (52.6% pS/T disordered compared to 27.8% npS/T sites, Fisher test p 8e-14, randomization test p
128 <1e-3, Figure 1). A more recent disorder predictor MFDp2 [27,28] also gives similar results
129 (43.7% pS/T disordered compared to 19.9 % npS/T sites, Fisher test p 6e-15, randomization test
130 p <1e-3, Figure 1). Disordered regions are also characterized by high irregular secondary
131 structure (coil regions). Thus, I tested whether pS/T residues are enriched in coil regions of
132 proteins. pS/T residues are more likely to occur in predicted coil regions compared to npS/T
133 residues (70.2% pS/T sites in coil compared to 55.7% npS/T sites in coil, Fisher test p 2e-5 and
134 randomization test p <1e-3, Figure 2). There is also depletion of pS/T residues in beta-sheet
135 regions (4.2% pS/T sites in sheet compared to 11.3% npS/T sites in sheet, Fisher test p 4e-4 and
136 randomization test p <1e-3, Figure 2), while no significant difference was found for helix regions

137 (22.8% pS/T sites in helix compared to 27.1 % npS/T sites in helix, Fisher test p 0.2 and
138 randomization test p 0.12, Figure 2).

139
140 Disordered regions evolve faster than ordered regions [41], but pS/T residues evolve slower than
141 npS/T in eukaryotes [42,43], so I tested whether *M. tuberculosis* pS/T and npS/T differ in
142 conservation among seven mycobacterial proteomes (see methods). Conservation of pS/T is not
143 significantly different from npS/T residues among these mycobacterial proteins (5.0 species vs.
144 5.2 species respectively using, Wilcox test p 0.92, number pS/T sites 103). Separately analyzing
145 disordered and ordered sites does not reveal significant difference in conservation between pS/T
146 and npS/T sites (Wilcox p 0.85 and 0.87 respectively). Thus pS/T are not more conserved than
147 npS/T among mycobacteria.

148
149 Pristic *et al.*[5] conducted *in vitro* phosphorylation of 13-mer synthetic peptides corresponding to
150 *in vivo* phosphorylation sites with different purified kinases. They could find phosphorylation of
151 about half of these peptides. I tested the whether different kinases have differential preference for
152 predicted disordered phosphoacceptors. I find that PknA has slightly higher preference for
153 disordered phosphoacceptors than other kinases (Figure 3); however the uncorrected Chi-square
154 test p -value is 0.04. Other kinases show uncorrected p -values > 0.05 .

156 Discussion

157
158 Here I show the enrichment of localized serine/threonine phosphosites in disordered regions of
159 proteins in *M. tuberculosis*. This preference is similar to that observed in eukaryotes. Association

between protein disorder and phosphorylation may offer similar advantages as those proposed in eukaryotes, including binding to multiple partners and transient mode of interaction [6,14–21], which are a prerequisite for regulatory interactions. Thus *M. tuberculosis* and eukaryotic serine/threonine phosphorylation dependent regulation may be more similar than generally appreciated. While most bacteria have low amount of protein disorder, *M. tuberculosis* has high disorder content. I suggest that high disorder content might be the reason for association between disorder and phosphorylation in *M. tuberculosis*. This suggestion is supported by the observation that other bacteria with high disorder content (*Streptomyces coelicolor* and *Halobacterium salinarum*) also show association between disorder and S/T phosphorylation, but bacteria with low disorder content do not (Supplemental Table 1) except for *Thermus thermophilus*, where enrichment of phosphorylation in disordered regions with marginal statistical significance was observed (Supplemental Table 1). Previously, a large fraction of phosphosites in *Thermus thermophilus* were observed in loop regions [44], albeit the statistical test for enrichment was not performed.

I find that PknA has a slightly higher preference for disordered phosphoacceptor sites on synthetic 13-mer substrate peptides under *in vitro* conditions than other kinases (Figure 3). However these results should be taken with the caveat that the structure of a peptide in *in vitro* conditions might be very different from the *in vivo* structure in the context of the full protein. Thus differential preferences of different kinases towards disordered substrates under *in vivo* conditions remain an open question.

I did not find pS/T sites to be more conserved than npS/T sites among mycobacteria, this suggests that phosphorylation network in mycobacteria evolves fast and may contribute towards niche specific adaptation. Important questions for future include, whether disordered and ordered S/T phosphosites are functionally different, and whether different kinases differ in their preference for disorder in their substrates under physiological conditions. Incorporation of disorder information might also be useful to predict novel S/T phosphosites [45], as has been shown in eukaryotes [6,46]. S/T kinases and their substrates have been linked to the survival, pathogenesis and virulence of *M. tuberculosis* [3,47,48], thus these finding may facilitate understanding the basic biology this deadly pathogen.

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References

1. Albuquerque CP, Smolka MB, Payne SH, Bafna V, Eng J, Zhou H (2008) A multidimensional chromatography technology for in-depth phosphoproteome analysis. *Mol Cell Proteomics* 7: 1389-1396.
2. Mijakovic I, Macek B (2012) Impact of phosphoproteomics on studies of bacterial physiology. *FEMS Microbiol Rev* 36: 877-892.
3. Cousin C, Derouiche A, Shi L, Pagot Y, Poncet S, Mijakovic I (2013) Protein-serine/threonine/tyrosine kinases in bacterial signaling and regulation. *FEMS Microbiol Lett* 346: 11-19.
4. Kobir A, Shi L, Boskovic A, Grangeasse C, Franjevic D, Mijakovic I (2011) Protein phosphorylation in bacterial signal transduction. *Biochim Biophys Acta* 1810: 989-994.

- 209 5. Prisic S, Dankwa S, Schwartz D, Chou MF, Locasale JW, Kang CM, Bemis G, Church GM, Steen
210 H, Husson RN (2010) Extensive phosphorylation with overlapping specificity by
211 Mycobacterium tuberculosis serine/threonine protein kinases. *Proc Natl Acad Sci U S A*
212 %20;107: 7521-7526.
- 213 6. Iakoucheva LM, Radivojac P, Brown CJ, O'Connor TR, Sikes JG, Obradovic Z, Dunker AK (2004)
214 The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res* 32:
215 1037-1049.
- 216 7. Collins MO, Yu L, Campuzano I, Grant SG, Choudhary JS (2008) Phosphoproteomic analysis of
217 the mouse brain cytosol reveals a predominance of protein phosphorylation in regions of
218 intrinsic sequence disorder. *Mol Cell Proteomics* 7: 1331-1348.
- 219 8. Gnad F, de Godoy LM, Cox J, Neuhauser N, Ren S, Olsen JV, Mann M (2009) High-accuracy
220 identification and bioinformatic analysis of in vivo protein phosphorylation sites in yeast.
221 *Proteomics* 9: 4642-4652.
- 222 9. Landry CR, Levy ED, Michnick SW (2009) Weak functional constraints on phosphoproteomes.
223 *Trends Genet* 25: 193-197.
- 224 10. Marchini FK, de Godoy LM, Rampazzo RC, Pavoni DP, Probst CM, Gnad F, Mann M, Krieger
225 MA (2011) Profiling the Trypanosoma cruzi phosphoproteome. *PLoS One* 6: e25381.
- 226 11. Amoutzias GD, He Y, Lilley KS, Van de PY, Oliver SG (2012) Evaluation and properties of the
227 budding yeast phosphoproteome. *Mol Cell Proteomics* 11: M111.
- 228 12. Uversky VN, Gillespie JR, Fink AL (2000) Why are "natively unfolded" proteins unstructured
229 under physiologic conditions? *Proteins* 41: 415-427.
- 230 13. Monastyrskyy B, Kryshchuk A, Moulton J, Tramontano A, Fidelis K (2014) Assessment of
231 protein disorder region predictions in CASP10. *Proteins* 82 Suppl 2:127-37. doi:
232 10.1002/prot.24391. Epub; %2013 Nov 22.: 127-137.
- 233 14. Singh GP, Ganapathi M, Dash D (2007) Role of intrinsic disorder in transient interactions of hub
234 proteins. *Proteins* 66: 761-765.
- 235 15. Tompa P, Szasz C, Buday L (2005) Structural disorder throws new light on moonlighting. *Trends*
236 *Biochem Sci* 30: 484-489.
- 237 16. Patil A, Nakamura H (2006) Disordered domains and high surface charge confer hubs with the
238 ability to interact with multiple proteins in interaction networks. *FEBS Lett* 580: 2041-
239 2045.
- 240 17. Dunker AK, Cortese MS, Romero P, Iakoucheva LM, Uversky VN (2005) Flexible nets. The roles
241 of intrinsic disorder in protein interaction networks. *FEBS J* 272: 5129-5148.
- 242 18. Wright PE, Dyson HJ (1999) Intrinsically unstructured proteins: re-assessing the protein structure-
243 function paradigm. *J Mol Biol* 293: 321-331.
- 244 19. Fink AL (2005) Natively unfolded proteins. *Curr Opin Struct Biol* 15: 35-41.

- 245 20. Dyson HJ, Wright PE (2005) Intrinsically unstructured proteins and their functions. *Nat Rev Mol*
246 *Cell Biol* 6: 197-208.
- 247 21. Tompa P (2002) Intrinsically unstructured proteins. *Trends Biochem Sci* 27: 527-533.
- 248 22. Ward JJ, Sodhi JS, McGuffin LJ, Buxton BF, Jones DT (2004) Prediction and functional analysis of
249 native disorder in proteins from the three kingdoms of life. *J Mol Biol* 337: 635-645.
- 250 23. Lew JM, Mao C, Shukla M, Warren A, Will R, Kuznetsov D, Xenarios I, Robertson BD, Gordon
251 SV, Schnappinger D, Cole ST, Sobral B (2013) Database resources for the tuberculosis
252 community. *Tuberculosis (Edinb)* 93: 12-17.
- 253 24. Dosztanyi Z, Csizmek V, Tompa P, Simon I (2005) IUPred: web server for the prediction of
254 intrinsically unstructured regions of proteins based on estimated energy content.
255 *Bioinformatics* 21: 3433-3434.
- 256 25. Dosztanyi Z, Csizmek V, Tompa P, Simon I (2005) The pairwise energy content estimated from
257 amino acid composition discriminates between folded and intrinsically unstructured
258 proteins. *J Mol Biol* 347: 827-839.
- 259 26. Walsh I, Martin AJ, Di DT, Tosatto SC (2012) ESpritz: accurate and fast prediction of protein
260 disorder. *Bioinformatics* 28: 503-509.
- 261 27. Mizianty MJ, Peng ZL, Kurgan L (2013) MFDp2 – accurate predictor of disorder in proteins by
262 fusion of disorder probabilities, content and profiles. *Intrinsically Disordered Proteins*
263 1:e24428.
- 264 28. Mizianty MJ, Uversky V, Kurgan L (2014) Prediction of intrinsic disorder in proteins using
265 MFDp2. *Methods Mol Biol* 1137:147-62. doi: 10.1007/978-1-4939-0366-5_11.: 147-162.
- 266 29. Combet C, Blanchet C, Geourjon C, Deleage G (2000) NPS@: network protein sequence analysis.
267 *Trends Biochem Sci* 25: 147-150.
- 268 30. Deleage G, Blanchet C, Geourjon C (1997) Protein structure prediction. Implications for the
269 biologist. *Biochimie* 79: 681-686.
- 270 31. Wolf YI, Koonin EV (2012) A tight link between orthologs and bidirectional best hits in bacterial
271 and archaeal genomes. *Genome Biol Evol* 4: 1286-1294.
- 272 32. Sievers F, Higgins DG (2014) Clustal Omega, accurate alignment of very large numbers of
273 sequences. *Methods Mol Biol* 1079:105-16. doi: 10.1007/978-1-62703-646-7_6.: 105-116.
- 274 33. Yang MK, Qiao ZX, Zhang WY, Xiong Q, Zhang J, Li T, Ge F, Zhao JD (2013) Global
275 phosphoproteomic analysis reveals diverse functions of serine/threonine/tyrosine
276 phosphorylation in the model cyanobacterium *Synechococcus* sp. strain PCC 7002. *J*
277 *Proteome Res* 12: 1909-1923.
- 278 34. Manteca A, Ye J, Sanchez J, Jensen ON (2011) Phosphoproteome analysis of *Streptomyces*
279 development reveals extensive protein phosphorylation accompanying bacterial
280 differentiation. *J Proteome Res* 10: 5481-5492.

- 281 35. Misra SK, Milohanic E, Ake F, Mijakovic I, Deutscher J, Monnet V, Henry C (2011) Analysis of
282 the serine/threonine/tyrosine phosphoproteome of the pathogenic bacterium *Listeria*
283 *monocytogenes* reveals phosphorylated proteins related to virulence. *Proteomics* 11: 4155-
284 4165.
- 285 36. Parker JL, Jones AM, Serazetdinova L, Saalbach G, Bibb MJ, Naldrett MJ (2010) Analysis of the
286 phosphoproteome of the multicellular bacterium *Streptomyces coelicolor* A3(2) by
287 protein/peptide fractionation, phosphopeptide enrichment and high-accuracy mass
288 spectrometry. *Proteomics* 10: 2486-2497.
- 289 37. Lin MH, Hsu TL, Lin SY, Pan YJ, Jan JT, Wang JT, Khoo KH, Wu SH (2009) Phosphoproteomics
290 of *Klebsiella pneumoniae* NTUH-K2044 reveals a tight link between tyrosine
291 phosphorylation and virulence. *Mol Cell Proteomics* 8: 2613-2623.
- 292 38. Aivaliotis M, Macek B, Gnad F, Reichelt P, Mann M, Oesterhelt D (2009) Ser/Thr/Tyr protein
293 phosphorylation in the archaeon *Halobacterium salinarum*--a representative of the third
294 domain of life. *PLoS One* 4: e4777.
- 295 39. Soufi B, Gnad F, Jensen PR, Petranovic D, Mann M, Mijakovic I, Macek B (2008) The Ser/Thr/Tyr
296 phosphoproteome of *Lactococcus lactis* IL1403 reveals multiply phosphorylated proteins.
297 *Proteomics* 8: 3486-3493.
- 298 40. Macek B, Mijakovic I, Olsen JV, Gnad F, Kumar C, Jensen PR, Mann M (2007) The
299 serine/threonine/tyrosine phosphoproteome of the model bacterium *Bacillus subtilis*. *Mol*
300 *Cell Proteomics* 6: 697-707.
- 301 41. Brown CJ, Takayama S, Campen AM, Vise P, Marshall TW, Oldfield CJ, Williams CJ, Dunker AK
302 (2002) Evolutionary rate heterogeneity in proteins with long disordered regions. *J Mol Evol*
303 55: 104-110.
- 304 42. Macek B, Gnad F, Soufi B, Kumar C, Olsen JV, Mijakovic I, Mann M (2008) Phosphoproteome
305 analysis of *E. coli* reveals evolutionary conservation of bacterial Ser/Thr/Tyr
306 phosphorylation. *Mol Cell Proteomics* 7: 299-307.
- 307 43. Gray VE, Kumar S (2011) Rampant purifying selection conserves positions with posttranslational
308 modifications in human proteins. *Mol Biol Evol* 28: 1565-1568.
- 309 44. Takahata Y, Inoue M, Kim K, Iio Y, Miyamoto M, Masui R, Ishihama Y, Kuramitsu S (2012)
310 Close proximity of phosphorylation sites to ligand in the phosphoproteome of the extreme
311 thermophile *Thermus thermophilus* HB8. *Proteomics* 12: 1414-1430.
- 312 45. Miller ML, Soufi B, Jers C, Blom N, Macek B, Mijakovic I (2009) NetPhosBac - a predictor for
313 Ser/Thr phosphorylation sites in bacterial proteins. *Proteomics* 9: 116-125.
- 314 46. Neduva V, Linding R, Su-Angrand I, Stark A, de MF, Gibson TJ, Lewis J, Serrano L, Russell RB
315 (2005) Systematic discovery of new recognition peptides mediating protein interaction
316 networks. *PLoS Biol* 3: e405.
- 317 47. Pereira SF, Goss L, Dworkin J (2011) Eukaryote-like serine/threonine kinases and phosphatases in
318 bacteria. *Microbiol Mol Biol Rev* 75: 192-212.

48. Av-Gay Y, Everett M (2000) The eukaryotic-like Ser/Thr protein kinases of Mycobacterium tuberculosis. Trends Microbiol 8: 238-244.

Figure Legends

Figure 1. Phosphorylated serine/threonine sites in *M. tuberculosis* are more likely to be

disordered. Both phosphorylated and non-phosphorylated serine/threonine sites are from the same set of proteins. The disorder was predicted using IUPred, Espiritz and MFDp2 methods. The Fisher test *p* values are 6e-8, 8e-14 and 6e-15 respectively. pS/T - phosphorylated serine/threonine, npS/T- non-phosphorylated serine/threonine.

Figure 2. Association between predicted secondary structure and phosphorylation of

serine/threonine sites in *M. tuberculosis*. Phosphorylated serine/threonine sites occur preferentially in coil regions, while being significantly depleted in sheet regions and show no significant difference in helix regions. The Fisher test *p* values are 2e-5, 4e-4 and 0.2 respectively. pS/T -phosphorylated serine/threonine, npS/T- non-phosphorylated serine/threonine.

Figure 3. Percentage of disordered phosphoacceptors are shown for different kinases. The

phosphorylation was carried out by purified kinases under in vitro conditions on synthetic 13-mer peptides corresponding to in vivo phosphorylation sites (Prisic et al.). Uncorrected Chi-square *p* values were significant (<0.05) only for PknA.

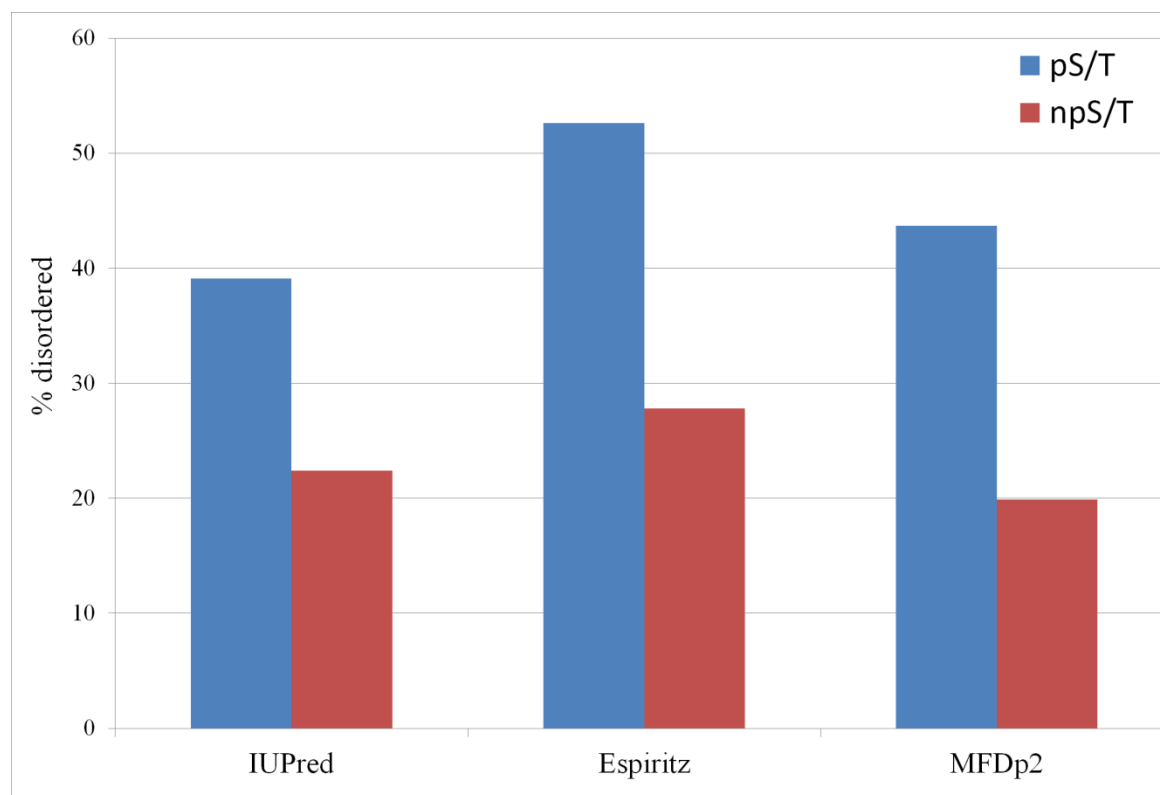


Figure 1

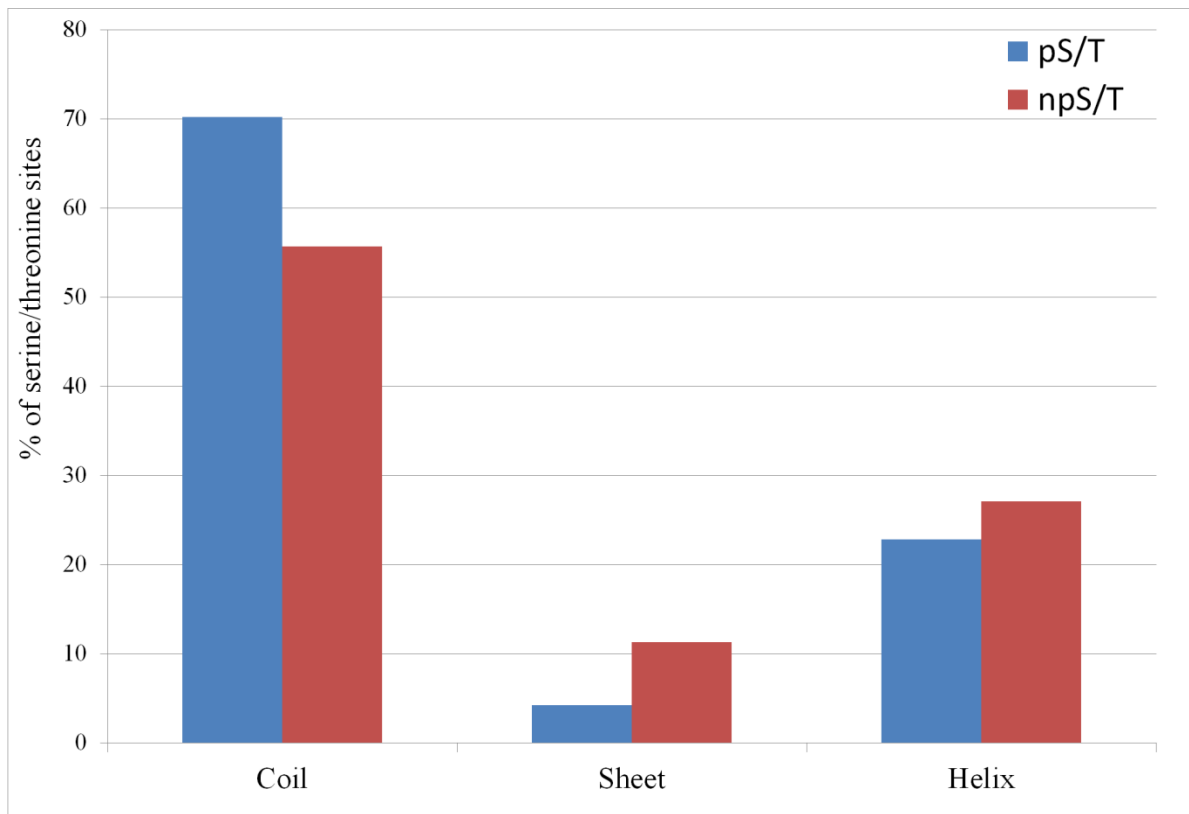


Figure 2

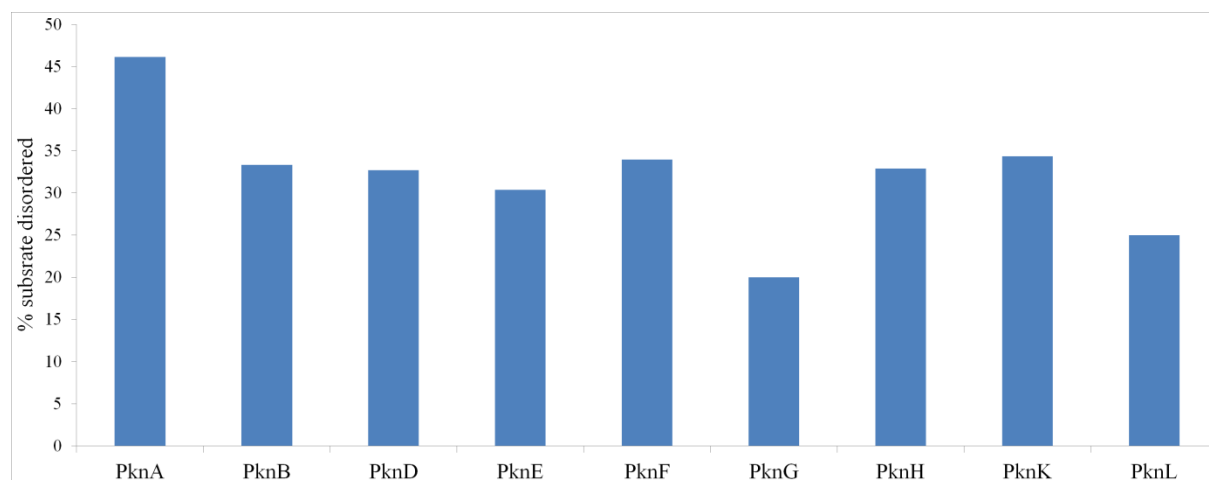


Figure 3