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# Découverte d'une nouvelle famille de protéine kinases bactériennes : mécanismes de fonctionnement et rôle cellulaire de YdiB, un archétype chez *Baccillus subtilis*

Hien-Anh Nguyen

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## THÈSE

Pour obtenir le grade de

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Présentée par

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préparée au sein de l'**Institut de Biologie Structurale J.-P. Ebel**,  
et du **CEA de Grenoble**  
dans l'**École Doctorale Chimie et Sciences du Vivant**

**Découverte d'une nouvelle famille de protéines kinases  
bactériennes :  
Mécanisme de fonctionnement et rôle cellulaire de  
YdiB, un représentant chez *B. subtilis***

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# UNIVERSITÉ DE GRENOBLE



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*A Benjamin, avec qui je me retrouve et me renouvelle*

« The only thing that interferes with my learning is my education »  
(Albert Einstein)

« For actually the earth had no roads to begin with,  
but when many men pass one way, a road is made »  
(Lu Hsun, My old home, 1921)

«... Nobody said it was easy  
No one ever said it would be so hard  
I'm going back to the start... »  
(Coldplay music band, The Scientist)

*After all..., tomorrow is another day...*  
(Margaret Mitchell, *Gone with the wind*, 1936)

Abstract :

Genome sequencing data has revealed genes encoding uncharacterized protein family UPF0079 which are exclusively found in bacteria; broadly distributed in this kingdom and possess an ATP-binding motif in their sequences. Biochemical characterization and physiological role elucidation of UPF0079 will undoubtedly increase our fundamental biology knowledge, and also remain a prerequisite towards the development of new antimicrobial compounds. Our investigation on YdiB, an archetype of this family in *Bacillus subtilis* revealed both autophosphorylating and protein phosphotransferase activities. The dual-specificity Ser/Thr and Tyr kinase activity of YdiB seems to require oligomerization is upregulated by basic molecule activators such as natural polyamines or poly-L-lysine. The 10 most conserved residues were studied to gain insights into molecular mechanism of the kinase YdiB. To characterize the function of phosphorylation events linked to YdiB, starting with the *B. subtilis ydiA-B-C-D-E* operon we showed that YdiB and YdiC function as cognate protein kinase/phosphatase towards two ribosome-related protein substrates YdiD and YdiE. Some co-localization between YdiB and ribosomes were observed. Furthermore, YdiB is capable of phosphorylating both the ribosomal 50S and 30S subunits as well as two ribosome-binding GTPases EngA and EngB. We also demonstrated that phosphorylated EngA by YdiB is an *in vitro* substrate of the phosphatase YdiC. Finally, based on the phosphoproteome of *Bacillus subtilis*, peptides mimicking the *in vivo* phosphorylation sites were used. Some of them were found to be phosphorylated *in vitro* by YdiB, including two peptides which belong to the superoxide dismutase SodA. The activity of purified SodA was then shown to be upregulated via phosphorylation by YdiB. We furthermore found that *B. subtilis* cells lacking *ydiB* become more sensitive to oxidative stress-causing agents such as paraquat or norfloxacin. We propose that *in vivo*, YdiB functions as a protein kinase involved in ribosome function in normal condition; and in protecting cells from oxidative stress damage.

Keywords : kinase, phosphatase, phosphorylation, YdiB, *Bacillus subtilis*, ribosome, oxidative stress

Résumé :

Les données de séquençage des génomes ont révélé la famille UPF0079 comprenant des protéines de fonction inconnue qui sont exclusivement présentes chez les bactéries; largement distribuées dans ce règne et possèdent le motif A de Walker dans leur séquence. La caractérisation biochimique et l'élucidation du rôle physiologique de cette famille contribueront à élargir nos connaissances en biologie fondamentale, et sont également un préalable vers le développement de nouveaux composés antimicrobiens. Notre étude sur YdiB, un archétype de cette famille chez *Bacillus subtilis* a révélé à la fois l'autophosphorylation de YdiB et son activité de protéine kinase. L'activité kinase de double spécificité Ser/ Thr et Tyr de YdiB semble nécessiter son oligomérisation et semble être stimulée par des molécules basiques telles que des polyamines naturelles ou la poly-L-lysine. Les 10 résidus les plus conservés chez cette famille ont été étudiés afin de mieux comprendre le mécanisme moléculaire de YdiB. Concernant la caractérisation fonctionnelle de la phosphorylation liée à YdiB, l'étude de l'opéron *ydiA-B-C-D-E* de *B. subtilis* nous a permis de montrer que YdiB et YdiC fonctionnent comme un couple de protéine kinase/phosphatase de deux protéines substrats dont les fonctions seraient liées aux ribosomes, YdiD et YdiE. Une co-localisation partielle entre YdiB et les ribosomes a été observée. En outre, YdiB est capable de phosphoryler des protéines ribosomiques appartenant aux deux sous-unités 50S et 30S, ainsi que deux GTPases liées aux ribosomes, EngA et EngB. Nous avons également démontré que EngA phosphorylée par YdiB est un substrat *in vitro* de la phosphatase YdiC. Enfin, basé sur le phosphoprotéome de *Bacillus subtilis*, des peptides mimant des sites de phosphorylation *in vivo* ont été utilisés. Certains d'entre eux ont été trouvés à être phosphorylés *in vitro* par YdiB. Deux de ces peptides appartient à la superoxyde dismutase SodA dont l'activité *in vitro* et après purification est régulée positivement via la phosphorylation par YdiB. Nous avons ensuite constaté que les cellules de *B. subtilis* dépourvues du gène *ydiB* sont plus sensibles aux agents oxydants tels que le paraquat ou la norfloxacin. Nous proposons que, *in vivo*, YdiB fonctionne comme une protéine kinase impliquée dans la fonction des ribosomes dans des conditions physiologiques normales, et son activité kinase contribuerait à protéger les cellules contre les dommages du stress oxydatif.

Mots-clés : kinase, phosphatase, phosphorylation, YdiB, *Bacillus subtilis*, ribosome, stress oxydatif



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# *Introduction*





## INTRODUCTION

If the year 1995 marked the first sequenced genome of a free-living organism, *Haemophilus influenzae*, at the end of 2011, full genome sequencing becomes normal, even 'blasé' science with the expected efficiency of 1000 genomes per month<sup>1</sup>. The major task that the scientific community is now facing is to turn data into knowledge<sup>2</sup>. In the first step, computational methods including sequence comparison, phylogenetic patterns and gene neighborhoods are inevitable to establish homolog network of each putative proteins in order to retrace its possible cellular function. However on average, there is no clear functional prediction for at least one-third of genes in most genomes<sup>3</sup>.

One of the most intriguing uncharacterized protein families regroups those found in many distantly related organisms. One such case includes a conserved family of small proteins, homologous to YjeE in *E. coli*, called UPF0079 in UniProtKB<sup>4</sup> or COG0802 in the COG database<sup>5</sup>. This family was mentioned for the first time by Galperin in 2001<sup>6</sup> with the purpose of illustrating the power and limitations of comparative genomics in deducing functions of uncharacterized proteins. Based on the conserved Walker A motif (Gx<sub>4</sub>GKT/S) which is a fingerprint to detect nucleotide-binding proteins, this protein family has been annotated as 'probable ATP-binding protein' in UniProtKB, and 'predicted ATPase or kinase' in COG database.

At the writing time of this manuscript, of the total 3237 UFP0079-homologs, it is noteworthy that still none of them comes from eukaryotes, rendering this family a promising target in antimicrobial drug development<sup>7</sup>.

The widespread distribution and conservation of this family of unknown function is implicit evidence for our lack of understanding of some basic cellular process. Bioinformatics can give some predicted clues for the general biochemical properties of these proteins. However, most of the time this approach is not sufficient and follow-up biochemical characterization is required to pinpoint the physiological function(s) of these new conserved proteins.

In 2002, Teplyakov & al.<sup>8</sup> solved the crystal structure of YjeE in *Haemophilus influenzae*, the first and still the only structure of UPF0079 family. This structure revealed a unique fold, evidence of a new class of nucleotide-binding proteins which possesses characteristics of both Kinase/GTPase and ATPase folds.

Since 2002, several groups have been reporting biochemical data on YjeE from *H. influenzae*, *E. coli* and for the past few years, our group has been focusing on YdiB, the counterpart from *B. subtilis*. An agreement was found on a very weak ATPase activity of these proteins<sup>8-10</sup>. The question of whether the real physiological function of this family is an ATPase arose when J. Karst in our group observed the *in vivo* oligomerization of YdiB which disfavored its inherently weak ATPase activity<sup>10</sup>.

In this manuscript we report that YdiB is in fact a new kinase with both autophosphorylating and protein phosphotransferase activities. To our knowledge, YdiB represents the first bacterial dual-specificity Ser/Thr and Tyr kinase family distinguishable from eukaryotic-like Ser/Thr/Tyr kinases. Along with the five major well-known prokaryotic protein kinase families (including Histidine kinases of two-component systems<sup>11-13</sup>, IDHK/P<sup>14-16</sup> and HPrK/P<sup>17-20</sup> discovered in the 1980's, eukaryotic like-Ser/Thr kinases of Hank's type<sup>21-23</sup> in the 1990s'; and the recently discovered branch of BYks<sup>24-27</sup> in the 2000's), the discovery of YdiB-like family by our group in collaboration with C. Grangeasse's team in the 2010's therefore opens a new chapter on prokaryotic protein kinases. Given the fact that this new family is broadly conserved in bacterial kingdom, we propose to name it Ubk for "Ubiquitous bacterial kinase".

Enzymatic characterization showed that oligomerization favors the kinase activity of YdiB and that this activity is increased by basic molecules such as natural polyamines or poly-L-lysine. We also provided insights into molecular mechanism of the kinase YdiB by studying the implication of the 10 most conserved residues on the autophosphorylating and protein phosphotransferase activities of YdiB.

The next important task is to characterize the function of phosphorylation events linked to YdiB, or in other words, we are dealing with the question: in which *in vivo* biochemical pathway does YdiB take place. Cellular partner "hunting" is one of the strategies to cope with this challenge. Starting with the *B. subtilis ydiA-B-C-D-E* operon we showed that YdiB, YdiC, YdiD and YdiE might participate in the same physiological pathway where YdiB and YdiC function as cognate protein kinase/phosphatase towards two putative ribosome-related protein substrates YdiD and YdiE. The link between YdiB and ribosome was furthermore strengthened by the phosphorylation by YdiB of ribosomal proteins of both 50S and 30S subunits as well as of two ribosome-binding GTPases EngA and EngB. We also demonstrated that phosphorylated EngA by YdiB is an *in vitro* substrate of the phosphatase YdiC. Given that either YdiBCDE, EngA and EngB are well conserved proteins, from an evolutionary point of view, these proteins might have co-evolved with ribosomes to secure the biogenesis and/or function of the protein-making machinery.

Using immunofluorescent technique, we demonstrated some co-localization between ribosomes and YdiB. This co-localization however, is not *stricto sensu* especially when cells were treated with antibiotics, since YdiB was also found in zones without ribosomes. We thus assume that YdiB might be a protein of multifunction that participates in more than one physiological pathway. We then showed that YdiB indeed is capable of phosphorylating a number of peptides which previously have been shown to be phosphorylated *in vivo* in *B.*

*subtilis* by Macek & al.<sup>28</sup> in 2007. In light of these results, a list of corresponding potential substrates of YdiB is now being under construction in order to confirm their *in vitro* phosphorylation by YdiB. We anticipate that YdiB is a protein kinase which may have global importance in *B. subtilis* thanks to its capacity of phosphorylating proteins involved in fundamental cellular processes such as protein synthesis, central carbohydrate metabolism, stress resistance, etc... Opening the YdiB puzzle might therefore leads to uncover a bigger picture where different separate pieces communicate via phosphorylation and signal transduction.

The last result reported in this manuscript focus on the involvement of YdiB in oxidative stress resistance via phosphorylation of the superoxide dismutase SodA. The superoxide scavenger capacity of this later protein against oxidative stress is upregulated via phosphorylation by YdiB. *B. subtilis* cells lacking *ydiB* therefore become more sensitive towards oxidative stress-causing agents such as paraquat or norfloxacin.

In conclusion, the finding of the protein kinase activity of YdiB, which possibly plays important role in ribosome function in normal physiological condition and in protecting cells from oxidative stress damage; as well as insights into its molecular mechanism involving oligomerization status and basic molecule effect, therefore contribute towards a more comprehensive understanding of this attractive yet previously poorly characterized protein.

# *Literature review*

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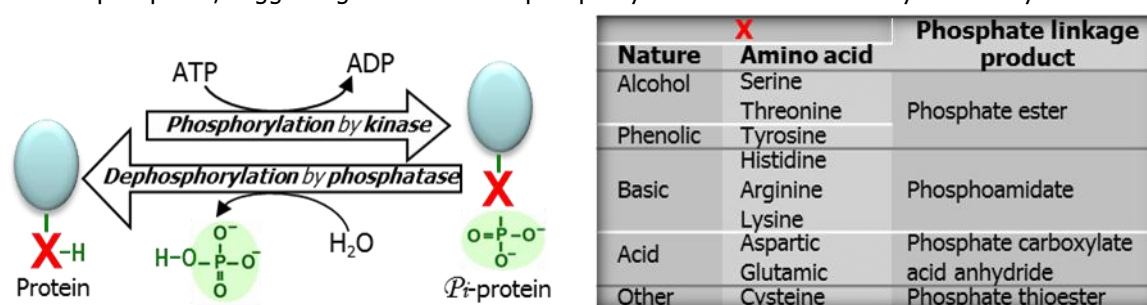
## I. LITERATURE REVIEW

### I.1. Protein phosphorylation in prokaryotes

#### I.1.1. Overview and Historical background

As early as the 19th century it was known that phosphates could be bound to proteins. Most examples of these phosphoproteins ( $P_i$ -proteins) were found in milk (caseins) and egg yolk (phosvitin) and were simply considered a biological method of providing phosphorus as a nutrient. Therefore, the existence of  $P_i$ -proteins was considered a consequence of metabolic reactions, and nothing more, for almost a century after their discovery<sup>29</sup>.

In the 1950s this all began to change as  $P_i$ -proteins emerged as key regulators of cellular life. An initiating factor of this emergence occurred in 1954, when an enzyme activity was observed that transferred a phosphate onto another protein<sup>30</sup> - a biological reaction called phosphorylation. The protein responsible was a liver enzyme that catalyzed the phosphorylation of casein and became known as a protein kinase (Fig. 1). A year later, the role of phosphorylation became more interesting as Fischer & Krebs<sup>31</sup>, and Wosilait & Sutherland<sup>32</sup>, showed that an enzyme involved in glycogen metabolism was regulated by the addition or removal of a phosphate, suggesting that reversible phosphorylation could control enzyme activity.



**Figure 1:** Reversible phosphorylation, and nature of Phosphate acceptor & linkage product<sup>33-37</sup>

Today, it is thought that about 30% of human proteins contain covalently bound phosphate, and more than 500 protein kinases and a third of that number of protein phosphatases are encoded by the human genome. Phosphorylation or dephosphorylation can affect the function of a protein in every conceivable way: increasing or suppressing activity, marking a protein for destruction (apoptose), allowing it to move from one sub-cellular compartment to another (motility, organelle trafficking, ion channels and membrane transport)<sup>38-41</sup>, or enabling it to interact with or dissociate from other proteins...<sup>42</sup>. The simplicity, flexibility and reversibility of this post-translational modification<sup>43</sup>, coupled with the availability of ATP as a phosphoryl donor, is presumably why it has been adopted to regulate so many biological processes.

Despite its obvious virtue as a vehicle for regulating protein function in eukaryotic cells, the existence and nature of protein phosphorylation-dephosphorylation in prokaryotes had long been the subject of controversy<sup>44</sup>. Early attempts during the 1960s and 1970s to detect protein phosphorylation in microbial organisms, by using the techniques that had been successful with mammals, proved negative. Many scientists concluded that protein phosphorylation-dephosphorylation was the exclusive province of higher organisms, a relatively late evolutionary invention devised to meet the special demands of organisms composed of multiple, differentiated cells - one for which simple organisms had no need and hence may do without<sup>43, 45</sup>.

It was only in the late 1970s, with the pioneering work of Wang and Koshland<sup>46</sup>, Garnak and Reeves<sup>14</sup>, and Manai and Cozzone<sup>47</sup>, which pointed out persuasive evidence that bacteria do contain specific protein kinases, confirming the universal virtue of protein phosphorylation. The following table summarizes the big landmarks in the history of prokaryotic protein phosphorylation.

**Table 1:** Historical landmarks in the elucidation of prokaryotic signaling

Year	Prokaryotic protein kinases/phosphatases
1969	-Kuo & Greengard <sup>48</sup> reported the phosphorylation of exogenous histones by a cAMP-dependent kinase in <i>E. coli</i> extracts. However, <i>E. coli</i> extracts harbor a <i>polyphosphate</i> kinase <sup>49</sup> and an <i>acyl-phosphate</i> kinase <sup>50</sup> that requires histones for maximum activity. The evidence of bacterial protein kinase was thus still unclear.
1973	-Rahmsdorf <sup>51</sup> showed that a cyclic nucleotide-independent protein kinase appeared in <i>E. coli</i> cells upon infection with bacteriophage T7. However, enzyme activity in uninfected cells is negligible. This protein kinase is, in fact, coded for by a specific viral gene, since its appearance is prevented by ultraviolet irradiation of the phage genome but not by that of the host genome <sup>52</sup> .
1978	-Wang & Koshland <sup>46</sup> provided <b>the first</b> persuasive evidence for <b>prokaryotic protein phosphorylation</b> . At least 4 proteins have been found to be serine/threonine-phosphorylated in <i>Salmonella typhimurium</i> .
÷	-Manai and Cozzone <sup>47</sup> also provided evidence for protein phosphorylation in <i>E. coli</i> . Phosphorylation occurs at the level of threonine and serine residues.
1979	-Garnak & Reeves <sup>14</sup> reported <b>the first endogenous substrate</b> for protein phosphorylation in bacteria. <i>E. coli</i> isocitrate dehydrogenase (IDH) was shown to be regulated by phosphorylation.
	-Alkaline sensitivity of the phosphate bonds in phosphorylated IDH suggests phosphoSer/Thr linkages <sup>53</sup> .

1980	-Spudich & Stoekenius <sup>54</sup> reported <b>the first</b> examples of <b>archaeal protein phosphorylation</b> . Two of them are light-regulated reversible phosphorylation – having its origin in retinal synthesis. -Acid and hydroxylamine resistance of the phosphate bonds in both proteins suggests phosphoSer/Thr linkages.
1981	-Wang & Koshland <sup>55</sup> detected <b>the first protein phosphatase</b> in <i>Salmonella typhimurium</i> .
1982	-Laporte & Koshland <sup>56</sup> characterized <b>the first bifunctional IDH kinase/phosphatase</b> (IDHK/P) in bacteria
1983	-Deutscher and Saier <sup>17</sup> found that the phosphotransferase activity of HPr was regulated by phosphorylation on serine, being reversibly controlled by a $P_i$ -(Ser)- <b>HPr-kinase</b> of 20 kDa and a $P_i$ -(Ser)- <b>HPr-phosphatase</b> of 70 kDa.
1986 ÷	-Nixon & al. <sup>11</sup> proposed a model for transduction of environmental signals by some pairs of regulatory gene products, which thereafter became the <b>TCS paradigm</b> ( <i>i.e.</i> two-component regulatory system).
1988	-Aspartate kinase activity of Histidine kinases <sup>57</sup> and auto-phosphatase activity of response regulators characterized <sup>58</sup> .
1987 ÷	-Stueland & al. <sup>59</sup> found an ATPase activity of IDHK/P
1988	-Cortay & al. <sup>60</sup> sequenced the gene <i>aceK</i> coding for IDHK/P
1988	-Cohen & al. <sup>61</sup> reported PP- $\lambda$ in bacteriophage $\lambda$ , <b>the first eukaryotic-like PPP-family protein phosphatase</b> .
1989	-Hurley & al. <sup>62</sup> solved the <b>structure</b> of <b>IDH</b> .
1990	-Guan & Dixon <sup>63</sup> discovered YopH from <i>Yersinia</i> , <b>the first eukaryotic-like PTP</b> family protein <b>phosphatase</b> .
1991	-Muñoz-Dorado & al. <sup>21</sup> discovered Pkn1, the <b>first eukaryotic-like Ser/Thr kinase</b> (eSTK) in <i>Myxococcus xanthus</i>
1993	-Min & al. <sup>64</sup> identified SpoIIAB from <i>B. subtilis</i> as a protein- Ser/Thr kinase with homology to TCS -Chang & al. <sup>65</sup> and Maeda & al. <sup>66</sup> discovered the first TCS in plant and in yeast. -Potts & al. <sup>67</sup> discovered IphP, the <b>first dual-specific PTP</b> -family protein phosphatase in <i>Nostoc commune</i> UTEX584.
1994	-Matsumoto & al. <sup>68</sup> reported the first evidence of phosphorylation of an exogenous protein (AfsR), by a 'eukaryotic'-like kinase (AfsK) from <i>Streptomyces coelicolor</i> .
1995	-Duncan & al. <sup>69</sup> identified SpoIIE from <i>B. subtilis</i> as <b>the first bacterial PPM</b> -family protein-Ser <b>phosphatase</b> .
1996	-Li & al. <sup>70</sup> characterized PtpA from <i>S. coelicolor</i> , as <b>the first bacterial LMW-PTP</b> -family protein-Tyr <b>phosphatase</b> . -Duclos & al. <sup>24</sup> detected <b>the first BY</b> (bacterial tyrosine) <b>autokinase</b> of about 81 kDa in <i>Acinetobacter johnsonii</i>
1997	-Two PPP-family protein phosphatases, PrpA and PrpB, characterized from <i>E. coli</i> <sup>71</sup> . -Grangeasse & al. <sup>72</sup> identified <i>ptk</i> as the gene encoding the first previously detected BY autokinase in <i>A. johnsonii</i>
1998	-Galinier & al. <sup>73</sup> identified <i>B. subtilis yvoB</i> as the gene encoding the ATP-dependent HPr-kinase
1999	-Ilan & al. <sup>74</sup> showed the first kinase activity from the BY-Ptk homolog, Etk towards exogenous substrate -Wu & al. <sup>75</sup> characterized <b>the first crosstalk</b> between <b>TCS</b> and bacterial <b>tyrosine</b> kinases, DivL from <i>C. crescentus</i> .
2001	-Fieulaine & al. <sup>18</sup> reported the <b>first crystal structure</b> of the <b>HPr Kinase/Phosphatase</b> (HPrK/P) in <i>Lactobacillus casei</i> , showing an ATP-binding loop of Walker A motif <sup>76,77</sup> .
2002	-Morona & al. <sup>78</sup> discover the <b>first PPM</b> -family protein <b>phosphatase</b> in <i>S. pneumoniae</i>
2003	-Ortiz-Lombardia & al. <sup>79</sup> presented <b>the first crystal structure</b> of an eSTK, PknB in <i>M. tuberculosis</i> .
2004	-Pullen & al. <sup>80</sup> described the <b>first crystal structure</b> of a eukaryotic-like <b>Ser/Thr protein phosphatase</b> , PstP/Ppp in <i>M. tuberculosis</i> <sup>81</sup> .
2005	-Nariya & Inouye <sup>22</sup> demonstrated the <b>first functional bacterial Ser/Thr kinase cascade</b> . The Pkn8–Pkn14 kinase cascade negatively regulates <i>mrpC</i> expression by phosphorylating MrpC during vegetative growth in <i>M. xanthus</i> . Not only MrpC is involved in eSTK signaling pathway, it is also regulated by the TCS MrpA-MrpB <sup>82,83</sup> .
2007 ÷	-Collins & al. provided <b>the first structural insights</b> of <i>E. coli</i> Wzc, a <b>BY kinase</b> , alone <sup>84</sup> or in complex <sup>25</sup> with the capsular polysaccharide translocon Wza.
2008	-Structures of <i>E. coli</i> Etk <sup>85</sup> (Proteobacteria) and <i>S. aureus</i> CapA/B <sup>86</sup> (Firmicutes) BY kinases were solved.
2010	-Zheng & Jia <sup>16</sup> solved <b>the first crystal structure</b> of <b>IDH K/P AceK</b> in its AMP-bound and AMP-free forms; alone or in complex with its IDH substrate.

### I.1.2. Phosphoproteomics in bacteria

Phosphoproteomics is a relatively new branch of proteomics that identifies, catalogs, and characterizes proteins containing a phosphate group as a post-translational modification.

Before 2007, traditional bacterial phosphoproteomics relied on 2D-gel separation of bacterial proteomes, <sup>32</sup>P radiolabelling or immunodetection, and detection of  $P_i$ -proteins by low-resolution mass spectrometry (MS). 128 phosphoproteins ( $P_i$ -proteins) from *E. coli*<sup>7</sup>, 29 from *B. subtilis*<sup>88</sup>, 41 from *C. glutamicum*<sup>89</sup> were found by such approach, but no phospho-sites ( $P_i$ -sites) were identified.

The combination of phosphoprotein enrichment and 2D-gel followed by MS analysis of trypsin digested spots however enabled identification of 36  $P_i$ -proteins including 3 on Tyr in *C. jejuni*<sup>90</sup>. Recently this approach allowed identification of 16  $P_i$ -sites from 63  $P_i$ -proteins of *M. pneumoniae*<sup>91</sup> and 51 novel  $P_i$ -proteins from *N. Meningitidis*<sup>92</sup>.

One of the advantages of 2D gel based phosphoproteomics is the relative ease with which changes in e.g. different growth conditions can be analyzed, but there are still some technical problems with this approach<sup>28,93,94</sup>. Starting from 2007, gel-free approach appeared, leading to the publication of site-specific Ser/Thr/Tyr bacterial phosphoproteomes for a number of species (*Tab. 3*). This approach, in short, involves digestion of crude extracts with endoprotease (e.g. trypsin) followed by phosphopeptide enrichment. The mixture is then separated by liquid chromatography (LC) that is coupled to a high resolution MS. A technique known as Stable Isotope Labeling by Amino acids in Cell culture (SILAC) is the latest development in the field of quantitative proteomics/ phosphoproteomics<sup>94</sup> that enables comparison of two strains (e.g. wild type and kinase or phosphatase mutant) or one strain cultured in two different growth conditions<sup>95</sup>.

The recent site-specific phosphoproteomes have greatly extended the list of known  $P_i$ -proteins and  $P_i$ -sites. Around 80÷100  $P_i$ -sites on average were reported in each bacterial phosphoproteome, except in *M. tuberculosis* with more than 500 sites, possibly due to its large STPKs number (11)<sup>96</sup>. These data nonetheless represent only a part of the global phosphorylation network owing to, on the one hand the dynamic, temporal



**Table 2: Phosphoproteomics, the systematic approach has been developed for almost one decade**

Bacterium	Year	Phosphoproteomic analysis
<i>C. glutamicum</i>	2003	Bendt & al. <sup>89</sup> used two methods: ( <sup>33</sup> P radiolabelling then autoradiography) and (immunostaining with phospho amino acid-specific antibodies), followed by 2D-gel and MS, to identify 41 phosphoproteins.
<i>C. bacterjejuni</i>	2007	Voisin & al. <sup>90</sup> analyzed the cytoplasmic fraction of <i>C. jejuni</i> by Fe-IMAC for phosphoprotein enrichment + 2D-gel for protein separation + in-gel tryptic digestion and MALDI-ToF MS for mass fingerprinting. The results revealed 58 phosphopeptides from 36 proteins.
<i>Bacillus subtilis</i>	Before 2006	-On establishing the complete genome of <i>B. subtilis</i> , Kunst & al. <sup>97</sup> showed 34 genes encoding response regulators (most of which have adjacent genes encoding histidine kinase to create two-component regulatory systems), and 11 aspartate phosphatase genes (involved in dephosphorylation of response regulators, that do not seem to have counterparts in Gram (-) bacteria such as <i>E. coli</i> ). -Only 16 $\Phi$ -sites on 8 of its proteins have been <i>in vitro</i> determined <sup>28</sup> , including PrkC (or YloP, 8 $\Phi$ -sites), SsbA, Idh, PtkA (or YwqD), Udg (also YwqF or TuaD, substrate of PtkA).
	2006	Lévine & al. <sup>88</sup> , by using <sup>32</sup> P radiolabelling with one-unit pH on 2D-gel combined with MS, indentified a total of 29 phosphoproteins, with 19 identified for the first time, however this study did not give any information on the location of $\Phi$ -sites.
	2007	-Macek & al. <sup>28</sup> performed gel-free analysis consisting of biochemical enrichment of phosphopeptides and high accuracy MS, identified 78 phosphoproteins with 78 $\Phi$ -sites (Ser:Thr:Tyr = 54:16:8). -Of 16 $\Phi$ -sites from 8 proteins reported so far, they detected 7 sites from 6 proteins. -Only 1 of 8 $\Phi$ -sites on PrkC was found. None of $\Phi$ -sites were found on SsbA, Idh and PtkA. By contrast, Ugd was identified, although Ugd phosphorylation <i>in vitro</i> is less efficient than that of PtkA.
<i>E. coli</i>	1986	Cortay & al. <sup>87</sup> , by using only <sup>32</sup> P radiolabelling with 2D-gel, showed 128 Ser/Thr/Tyr protein spots. They were mainly located in the cytosolic fraction of cells. However most of them were only described in terms of pI and molecular weight <sup>87</sup> and, surprisingly, were never identified <sup>98</sup> .
	1986 ÷ before 2008	-No homologs of eukaryotic Ser/Thr kinases had so far been found in <i>E. coli</i> , except a preliminary description of a protein kinase C-like activity <sup>99</sup> . <i>E. coli</i> possesses 2 well characterized Ser/Thr kinases: the IDHK/P <sup>100</sup> and the YihE kinase <sup>101</sup> . Other <i>E. coli</i> proteins were also reported to autophosphorylate on Ser/Thr residues, including DnaK, the molecular chaperone heat-shock protein <sup>102,103</sup> ; Era, an essential Ras-like GTPase <sup>104</sup> , and the nucleotide diphosphate kinase (NDK) <sup>105</sup> . - <i>E. coli</i> possesses 3 BY-kinases, BipA <sup>93,106,107,108</sup> , Wzc and Etk <sup>84,108,109</sup> , which are capable of auto- and substrate phosphorylation. -At least 62 open reading frames (ORFs) were identified as putative members of the two-component signal transducers in <i>E. coli</i> . Among them, 32 were identified as response regulator and 23 others as orthodox sensory kinases. In addition, <i>E. coli</i> has five hybrid sensory kinases <sup>110</sup> . -Available biochemical data include 12 phosphorylation sites on 6 proteins in <i>E. coli</i> <sup>107,111</sup> .
	2008	Macek & al. <sup>98</sup> performed the same method used in 2007 <sup>28</sup> and identified 79 proteins with 81 $\Phi$ -sites (Ser:Thr:Tyr = 55:19:7).
	2009	Soung & al. <sup>112</sup> used either phosphorylation specific visualization techniques, (Pro-Q diamond staining) or specific phospho-Ser, Thr, Tyr antibodies followed by MS/MS after phosphopeptide enrichment through immobilized Metal Affinity Chromatography and by peptide fractionation through Strong cation eXchange Chromatography (SXC), to analyze phosphorylated proteins in <i>E. coli</i> ribosomes. Of the 54 proteins in the 70S ribosome, they found 24 to be steady-state phosphorylated. 9 of them belong to the small subunit 30S, the rest are in the large subunit 50S. Only 5 of these ribosomal phosphoproteins were previously detected in the phosphoproteome by Macek & al. <sup>98</sup> .
<i>Lactococcus lactis</i>	2008	-Soufi & al. <sup>113</sup> performed the same method used by Macek & al. <sup>28,98</sup> to identify 73 $\Phi$ -sites distributed over 63 different proteins. -The presence of several multiply phosphorylated proteins, as well as over-representation of phospho-Thr seems to be the distinguishing features of the <i>L. lactis</i> phosphoproteome.
<i>Klebsiella pneumoniae</i>	2009	-Lin & al. <sup>114</sup> developed the technique previously used by Soung & al. <sup>112</sup> through a gel-free analysis consisting of peptide fractionation by SXC + phosphopeptide enrichment by TiO <sub>2</sub> affinity and by phospho-tyrosine specific antibodies + LC-LTQ-OrbitrapMS/MS analysis. They identified 81 proteins with 93 $\Phi$ -sites (Ser:Thr:Tyr:His:Asp= 29:14:24:12:14). -Using phospho-tyrosine specific antibodies for enrichment led to the identification of 17 additional Tyr phosphorylated peptides out of 24 in total.
<i>Pseudomonas spp</i>	2009	-Ravichandran & al. <sup>115</sup> developed a novel phospho-enrichment method using aliphatic Hydroxy Acid-Modified Metal Oxide Chromatography (HAMMOC) with titania and zirconia. -By Ti/Zr HAMMOC-based nanoLC-MS approach, they founded 53 $\Phi$ -sites of <i>P. putida</i> (Ser:Thr:Tyr = 28:21:4) and 55 $\Phi$ -sites of <i>P. aeruginosa</i> (Ser:Thr:Tyr = 29:18:8) -Interestingly, the GTP-binding protein EngA was found to be phosphorylated in <i>P. putida</i> at Ser-34 in this study.
<i>Streptococcus pneumoniae</i>	2010	-Sun & al. <sup>116</sup> used TiO <sub>2</sub> enrichment and LC-MS/MS to reveal 102 unique phosphopeptides corresponding to 84 proteins and 163 $\Phi$ -sites with distributions of (Ser:Thr:Tyr = 48:45:9) -A striking characteristic of <i>S. pneumoniae</i> phosphoproteome is the large number of multiple species-specific phosphorylated sites, indicating that high level of protein phosphorylation may play important roles in regulating many metabolic pathways and bacterial virulence. EngA was found to be phosphorylated in <i>S. pneumoniae</i> at Thr-5 in this study.
<i>M. pneumoniae</i>	2010	-Schmidl & al. <sup>91</sup> used 2D-gel followed by MS to identify 16 $\Phi$ -sites (Ser:Thr = 8:8) from 63 proteins.
<i>S. coelicolor</i>	2010	-Parker & al. <sup>117</sup> performed gel-free analysis consisting of protein and peptide fractionation by IEF and SXC chromatography respectively + phosphopeptide enrichment by TiO <sub>2</sub> affinity chromatography + phosphopeptide identification by LC-ESI-LTQ-Orbitrap MS. The result revealed 46 novel $\Phi$ -sites on 40 proteins with a Thr-preferred profile (Ser:Thr:Tyr = 16:24:6)
<i>Mycobacterium tuberculosis</i>	Before 2010	-If <i>B. subtilis</i> and <i>E. coli</i> have each more than 30 two-component systems (TCS) <sup>97,110</sup> , <i>M. tuberculosis</i> has only 11 complete pairs of sensor histidine kinases and response regulators, and a few isolated kinase and regulatory genes <sup>118</sup> . -This relatively paucity of TCS is offset by the presence of many eukaryotic-like Ser/Thr protein kinases (eSTKs) which function as part of a phosphorelay system. <i>M. tuberculosis</i> has only 3 eukaryote-like protein phosphatase (PstP, PstA, PstB) but 11 eSTKs (PknA to PknL) <sup>119</sup> . - <i>Mycobacteria</i> with larger genomes such as <i>M. marinum</i> or <i>M. smegmatis</i> STPKs outnumber TCS, suggesting that the bulk of signal transduction is via Ser/Thr phosphorylation <sup>120</sup> .
	2010	-Prisic & al. <sup>121</sup> used a gel-free approach followed by LC-MS/MS to identify 516 $\Phi$ -sites inside 301 with a Thr-preferred profile (Ser:Thr = 60%:40%). -The motif X <sub>aa</sub> TX(X/V) $\Phi$ (P/R)I (where $\alpha$ is an acidic residue and $\Phi$ a large hydrophobic residue) was proposed to be the specific recognition substrate-kinase motif for 6 eSTKs (PknA, B, D, E, F, H). This common motif explains why multiple kinases can target a single protein, (e.g GarA and Rv1422).
<i>Neisseria</i>	2011	Bernardini & al. <sup>92</sup> , by using 2D-gel with Pro-Q diamond stain and MALDI-ToF/MS, revealed 51 novel phosphoproteins

Color code: Green (Latest results in each organism), Gray (Studies using gel-free approach)



and environmentally dependent *kinome*<sup>(\*)93</sup>, on the other hand the technical imperfections. However, in term of evolution, one can anticipate right now a lack of (or extremely low) conservation of  $P_i$ -sites<sup>96 122</sup>, since only one single protein, the phosphosugar mutase (ManB in *M. pneumoniae* or GlmM in *B. subtilis*), is phosphorylated on a conserved Ser residue in all studied organisms, from archaea and bacteria to man. For most other proteins (e.g. DnaK or Hsp70), even if they are phosphorylated in different species, the actual  $P_i$ -sites are different. This suggests that site-specific phosphorylation is a form of adaptation of the bacteria to the specific needs of their particular ecological niche<sup>113, 122</sup>.

An alternative to experimental identification of  $P_i$ -sites is in silico prediction. Originally trained on eukaryotic data, the most widely phosphorylation predictor NetPhos fell short of performance on bacterial proteins (e.g. when it was evaluated on the published phosphoproteome of *B. subtilis*<sup>94</sup>). This led to the development of a bacteria-specific version NetPhosBac<sup>123</sup> that out-competed the eukaryotic predictors as well as the bacterial - specific mode of DISPHOS predictor<sup>96</sup>. NetPhosBac is currently dedicated to Ser and Thr phosphorylation due to the limited number of known Tyr  $P_i$ -sites, but it is only a question of time before enough data for a Tyr specific version is available.

In term of functional insights obtained from phosphoproteomic studies,  $P_i$ -proteins are seemingly over-represented in central carbon metabolism (especially among glycolytic enzymes)<sup>28,94,98</sup>, as well as in house-keeping processes including the virulence functions of bacterial pathogens<sup>93</sup>. But the physiological roles of these phosphorylation events are still largely unknown. Furthermore, a number of  $P_i$ -proteins of unknown function were also comprised in each published phosphoproteome. To link protein kinases and their substrates more than ever remains the main challenge ahead. At this point, along with the improvement of analytical tools (e.g. SILAC), one can expect a new wave of bacterial kinomes (including wild type as well as kinase/phosphatase-knockout strains) in different growth conditions. Such data should allow us to infer connections between kinases/phosphatases and their substrate and hopefully their physiological role(s), a stepping stone towards bacterial systems biology<sup>96</sup>. Interestingly, a recent study applied this approach in *M. pneumoniae* with the surprising conclusion that only 5 out of 63 phosphoproteins are targeted by the two known kinases pointing towards the presence of novel classes of kinases<sup>91</sup>.

### I.1.3. Classification of bacterial protein kinases

The hypothesis towards the presence of novel kinases undetected by analytical phosphoproteomic tools has become more and more apparent. The lack of a significant bacterial protein kinase classification system based on conserved features and deduced phylogeny of the catalytic domains (as the eukaryotic one established by Hanks & al.<sup>124</sup>), in fact does not help to accelerate the characterization of these kinases of unknown function. The first framework for classification of prokaryotic Ser/Thr kinases was proposed by Tyagi & al.<sup>125</sup>, but is still far from convenient utilization.

Traditional classification proposed by Hunter<sup>34</sup> (on the basis of phosphate acceptor amino acids, *fig. 1*) in combination with the kinase sequence analysis (based on the presence of the phosphate-binding motif Gx<sub>4</sub>GKT[S], so-called Walker A motif<sup>76,77</sup>) will be developed in the *table 4*, on purpose of systematization of representatives of bacterial protein kinases. Only kinases which do not contain a Walker A motif will be shortly described inside this section, aiming to show the highly divergence phosphorylation in bacteria, which is in fact a proper versatile attribute in order to adapt to environmental stimulus, but not acquired from eukaryote by horizontal gene transfer. Other protein kinases containing the Walker A motif (including YdiB family which is present exclusively in prokaryote but not in eukaryote - objective of this thesis) will be introduced in I.2.

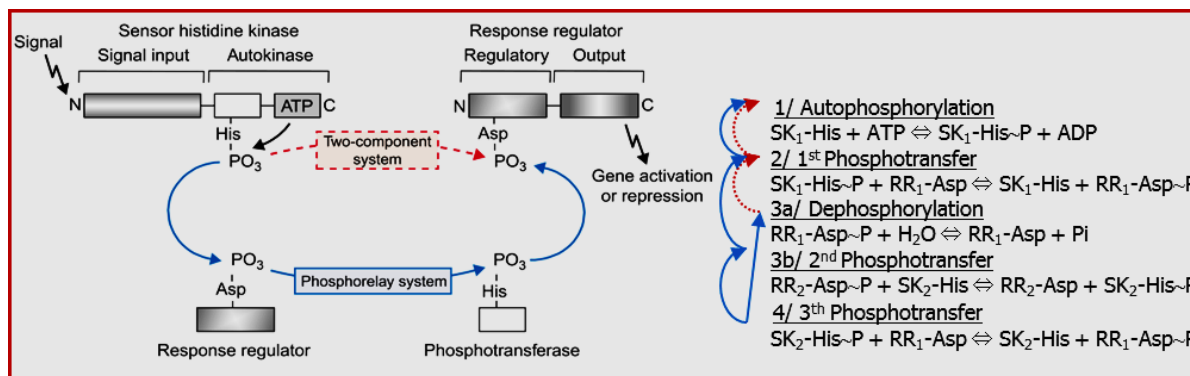
**Table 3:** Classification of bacterial kinases by kinase sequence & phosphate acceptors

Phosphate acceptor	Main bacterial kinase families	Walker A motif
Histidine/Asparate	Two-component systems (TCS)	
	Isocitrate Dehydrogenase Kinase/Phosphatase (IDHK/P)	
Serine/Threonine	Two-component system-like Ser/Thr kinase	-
	Eukaryotic-like Ser/Thr kinases (eSTK)	
	Histidine Protein Kinase/Phosphatase (HPrK/P)	
Tyrosine	Bacterial Tyrosine Kinases (BY)	+
	Two-component system-like Tyr kinase (DivL)	
	Eukaryotic-like Tyr kinases (Mask & WaaP)	
	Haloacid dehalogenase-like hydrolase Tyr kinase (PtkA)	

#### I.1.3.1. Two-component systems (TCS)

The first model of TCS was proposed by Nixon & al.<sup>11</sup> in 1986, based on the chemotaxis previously observed in several *E. coli* sensory transducers<sup>91, 92</sup>. It has become clear that TCS is widespread, although not ubiquitous, in bacteria and archaea (30 to 40 complete TCS was found in *E. coli*, *B. subtilis*, or *Synchosystis* ssp., more than 100 in *Nostoc punctiformis*<sup>128</sup>, but none in *Mycoplasma fenitaliul* or *Methanococcus jannaschii*<sup>129</sup>). TCS was found lately in plant<sup>65</sup> and in yeast<sup>66</sup>, but is seemingly absent in animals, worm and fly<sup>129,12</sup>.

TCS consists of a signal recognition sensor kinase (SK) that autophosphorylates on a Histidine, usually in response to the presence of a signal; and a response regulator (RR) transcription factor that activates or represses gene expression when phosphorylated on an Aspartate by the cognate sensor kinase.



**Figure 2:** *Two-component & Phosphorelay transduction system* (based on <sup>97,98,13</sup>)

SK generally contains a signal input domain coupled to an autokinase domain (which can be divided into a Histidine-phosphotransferase subdomain and an ATP-binding subdomain distinguishable from Walker A motif<sup>131</sup>, Fig. 5). RR has a regulatory domain that controls the activity of the output domain. Detection of the stimulus by the SK induces the transfer of  $\gamma$ -phosphate to His residue. The phosphoryl group from the His of the SK will be transferred to the Asp of the regulatory domain of RR<sup>128</sup>. Finally, the auto-dephosphorylation of this RR<sup>96, 97</sup> will return to the initial form by releasing enough energy to trigger on/off the output signal (Fig. 2).

More-complex variants of TCS, termed phosphorelays, are used in bacteria for pathways responding to multiple signal inputs<sup>133</sup>. Phosphorelays in fact are four-component systems with an additional regulatory domain and a phosphotransferase subdomain. The signal transduction pathway directs phosphoryl transfer in H-D-H-D sequence. In the sporulation phosphorelay, these domains occur on separate proteins<sup>134-136</sup>, but in other phosphorelays, one or more subdomains might be attached to the sensor kinases as a polydomain protein<sup>12,128,130,134,135,137-139</sup>.

**Table 4:** *Bacterial Phosphorelay architectures* (updated from <sup>140</sup> and <sup>141</sup>)

Phosphorelay architectures	Representative	Description
	EnvZ-OmpR ( <i>E. coli</i> )	Typical TCS, E is an effector protein mediating DNA binding and transcriptional control of target genes <sup>142-144</sup>
	BvgS-BvgA ( <i>B. pertussis</i> )	The first regulator domain RR <sub>1</sub> and the second Histidine-phosphotransfer domain HPT, are fused to the hybrid SK
	KinA-Spo0F-Spo0B-Spo0A ( <i>B. subtilis</i> )	Typical phosphorelay as described above, except that Spo0B is not a typical HPT domain <sup>145, 146</sup>
	CheA-CheY ( <i>E. coli</i> )	The sensory receptor is detached from the His-kinase CheA. The RR consists of only a regulatory domain CheY <sup>107, 108</sup>

25 years have passed since the first model proposed on the basis of NtrB/NtrC pair in *E. coli*. Up to now what do we know and do not know about this TCS-phosphorelay system in bacteria?<sup>130, 139</sup>

#### We know:

- TCS is strain specific but also conserved among strains to respond to the same type of stimulus<sup>149</sup>. TCS is widely distributed but not universal<sup>94,129</sup>; 2 different mechanisms were proposed to explain the evolution of TCS through recruitment & co-evolution<sup>149</sup>.
- TCS participate in lots of pathways in response to a myriad of signals<sup>132,150</sup>.
- The structure of regulator and output domains of RR, the structure of ATP domains and phosphotransferase domains of histidine kinases<sup>129</sup>.
- A limited appreciation of the mechanism of phosphotransfer between the two proteins<sup>128</sup>.

#### We do not know:

- The structure of membrane-based signal input domains for the majority of kinases. Is membrane location important for their function?
- How signals activate kinases? How regulator domains control output domains? (except few examples)
- How phosphorylation interferes with this control, although we have some suspicions about how this works, through several modeling studies<sup>139</sup>.
- What are the functional differences between simple single-step TCS and the various phosphorelays? What are the general principles that may account for the use of one architecture over another?

#### Future research:

- Modelization and experimental validation in larger-scale.
- Interactions between TCS and other regulatory networks in the cell.
- Development of new methods to follow histidine kinase and response regulator activity *in vivo*<sup>139</sup>.
- Two-component systems and phosphorelays as targets for therapeutic intervention<sup>13</sup>.

### I.1.3.2. Eukaryotic-like Ser/Thr kinases

The eukaryotic Ser/Thr protein kinase paradigm (Hanks-type)<sup>124</sup> was first challenged in 1991 by *pkn1* from *Myxococcus xanthus* – an ORF whose predicted product Pkn1 resembles eukaryotic Ser/Thr kinases<sup>21</sup>. Three years after, the first evidence of phosphotransfer to a protein substrate (AfsR) by a 'eukaryotic'-like kinase (AfsK) from *Streptomyces coelicolor*, was reported<sup>68</sup>. With the advent of genome sequencing, scientists began to trace the distribution of ORFs encoding deduced protein kinases<sup>111,151,152</sup>. Of 27 bacterial genomes analyzed, 85% contained ORFs for potential eukaryotic-like protein kinases<sup>43</sup>. The following *Tab. 5* updates 33 biochemically studied Hanks-type Ser/Thr kinases from 13 bacteria.

**Table 5: Bacterial eSTKs and their substrates** (updated from <sup>23, 153, 154, 155</sup>)

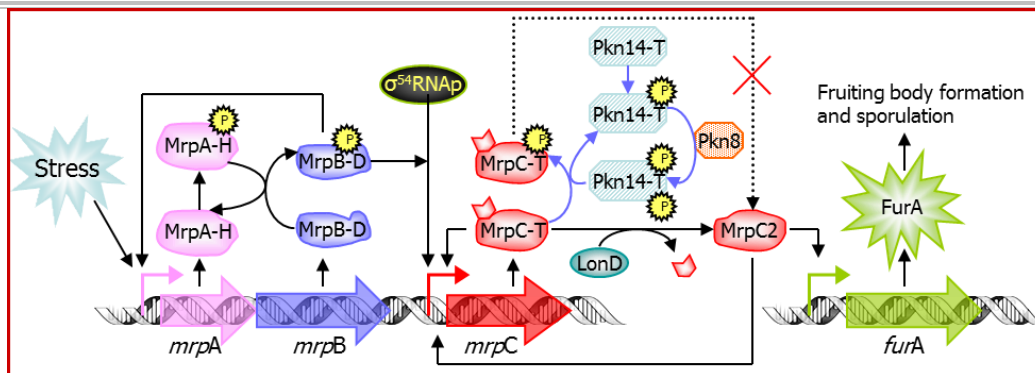
<i>Bacillus subtilis</i>					Function	Methodology-References	Year								
Substrates	PrkC	PrkD	YabT	Comment											
YabT			+		Sporulation, biofilm, germination, cell wall	KA, MS, 3D structure <sup>156-159</sup>	2002								
AlsD	+			PrkC and PrkD are respectively so-called YoiP and YbdM	$\alpha$ -Acetolactate decarboxylase; central metabolism	Kinase assay (KA) and Mass spectrometry (MS) <sup>160</sup>	2010								
GlnA	+	+			Glutamine synthetase; central metabolism										
Icd	+	+													
Hpr	+				Central metabolism										
YwjH	+				Kinase, phosphotransferase system										
CpgA	+				Transaldolase; central metabolism	KA, mutagenesis	2009								
YezB	+				GTPase; peptidoglycan sacculus deposition	Phosphoamino acid analysis (PAA), 2D gel <sup>161</sup>									
EF-Tu	+				Stressome protein										
EF-G	+				GTPase; Elongation factor; protein translation	Immunoprecipitation, KA <sup>157,162,163</sup>	2002								
PrkD		+			GTPase; Elongation factor; protein translation	<sup>156, 160</sup>									
DegS		+	+		Cytosolic sensor kinase of TCS of DegS/U, swarming	KA, mutagenesis <sup>164</sup>	2011								
<i>Chlamydia trachomatis</i>					Function	Methodology-References	Year								
Substrates	Pkn1	PknD		Comment											
Pkn1	+	+			Unknown	Bacterial two-hybrid immunoprecipitation, KA <sup>165</sup>	2003								
IncG	+				Virulence										
PknD		+			Unknown										
<i>Corynebacterium glutamicum</i>					Function	Methodology-References	Year								
Substrates	Kinases Pkn				Comment										
	A	B	G	L											
MurC	+				Both FtsZ and OdhI are substrates of the phosphatase Ppp	KA, MS, mutagenesis <sup>166</sup>	2009								
FtsZ	+	+	+			Cell wall synthesis									
PknG	+					Cell division		KA, MS, <i>in vitro</i> and <i>in vivo</i> 2D gel <sup>167, 168</sup>							
OdhI	+	+	+	+		Soluble eSTK									
					FHA protein, Glutamate catabolism										
<i>Mycobacterium tuberculosis</i>					Function	Methodology-References	Year								
Substrates	Kinases PknA to PknL														
	A	B	D	E	F	G	H	I	J	K	L				
PknA		+											Cell elongation / division	KA <sup>169</sup>	2009
PknB		+	+										Elongation/division, peptidoglycan synthesis	KA, MS, 3D structure <sup>169-171</sup>	2003
PknD													$\beta$ -propeller, PQQ domain, phosphate transport	KA <sup>172</sup>	1997
PknF						+							Membrane transporter	KA <sup>173</sup>	2004
PknG							+						Trx & TPR motif, glutamate metabolism	KA, MS <sup>174</sup>	2008
PknH								+					AfsK like, arabian metabolism, NO stress	KA, PPA <sup>175</sup>	2003
PknI									+				Abnormal Asn in active site, cell division	KA <sup>176,177</sup>	2004
PknJ										+			-	KA, PPA, mutagenesis <sup>178-180</sup>	2006
PknK											+		Transcription, 2 <sup>nd</sup> metabolism	KA, PPA <sup>179,181</sup>	2009
EmrB	+	+						+			+		Arabinan synthesis, cell wall	KA, PPA, Phosphor-chip <sup>175,180</sup>	2003
PBPA		+											Cell wall synthesis	KA, MS, mutagenesis <sup>182</sup>	2006
GlmU	+	+											Cell wall (peptidoglycan) synthesis	KA, MS, mutagenesis <sup>171</sup>	2009
DacB1							+						Penicillin-binding protein, cell wall	KA, MS, mutagenesis <sup>171</sup>	2009
FabH	+							+	+	+	+		Mycolic acid pathway; cell wall biosynthesis	<i>In vivo</i> KA, MS, mutagenesis <sup>183</sup>	2009
FabD	+	+									+		Mycolic acid biosynthesis	KA, PAA <sup>179</sup>	2006
KasA	+	+	+	+	+						+		Mycolic acid biosynthesis		
KasB	+	+	+	+	+						+		Mycolic acid biosynthesis		
MabA	+	+	+	+							+		Mycolic acid biosynthesis	KA, MS, mutagenesis <sup>184</sup>	2010
MmA4											+		Mycolic acid biosynthesis	KA, Phospho-chip, MS <sup>180</sup>	2010
PepE											+		DNA-binding protein, cell wall/cell division		
FibA	+												Cell division under H <sub>2</sub> O <sub>2</sub> oxidative stress	<i>In vivo</i> KA, MS, mutagenesis <sup>185</sup>	2010
FtsZ	+												Cell division	KA, MS <sup>185,186</sup>	2006
MurD	+												Cell division	KA <sup>187</sup>	2008
Wag31	+												Cell division, orthologue of DivIVA	<i>In vivo</i> KA, MS, mutagenesis <sup>169</sup>	2005
Mmpl7			+										Membrane transporter; resistance, cell division	<i>In vivo</i> 2D gel, MS <sup>188</sup>	2006
Rv1747		+		+	+								Putative ABC transporter	KA <sup>173,189</sup>	2004
Rv0020c		+			+								FHA-containing protein	KA <sup>189</sup>	2005
Rv2175											+		Dipeptidase, DNA-binding protein	2D gel, PAA, MS, KA <sup>190,191</sup>	2008
Rv1422	+	+											-	<i>In vivo</i> KA, MS, mutagenesis <sup>169</sup>	2005
PykA											+		Pyruvate kinase A, Glycolysis	KA <sup>178</sup>	2009
GarA	+					+							Glycogen recycling, tricarboxylic acid cycle	KA, MS, SPR <sup>174</sup>	2008
SigH	+												Oxidative, nitrosative, heat stress $\sigma^H$ factor	<i>In vivo</i> KA, MS, mutagenesis <sup>192</sup>	2008
RshA	+												Oxidative, nitrosative, heat stress anti- $\sigma^H$ factor		
Rv0156			+										Anti-anti-sigma factor	<i>In vivo</i> KA, MS, mutagenesis <sup>193</sup>	2007
VirS											+		Transcriptional regulator	KA, PPA <sup>179,181</sup>	2009
Rv0681						+							TetR class transcription factor	KA, mutagenesis <sup>194</sup>	2007
DosR						+							Dormancy, response regulator of TCS DosS/R	KA, MS, mutagenesis <sup>195</sup>	2010

GroELI										+	+	+	+	+	+	+	+	+	+				
<b><i>Mycoplasma pneumoniae</i></b>										Heat shock protein										<i>In vivo</i> KA, MS, mutagenesis <sup>196</sup>	2009		
<b>Function</b>																				<b>Methodology-References</b>	<b>Year</b>		
Substrates Kinases PrkC																				Pro-Q Diamond staining of cell lysates <sup>197</sup>			
HMW1-3										+										Cytadherence proteins			
MPN474										+										Surface protein			
P1										+										Adhesion			
<b><i>Myxococcus xanthus</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrates Kinases Pkn1 to Pkn14																							
										1 2 3 4 5 6 8 9 14													
Pkn1										+										Proper differentiation		<sup>21, 198</sup>	1991
Pkn2										+										Negative for vegetative cell, necessary for spore		<sup>199-201</sup>	1995
Hu $\alpha$										+										Histone-like DNA binding protein		KA, mutagenesis <sup>202</sup>	2000
$\beta$ Lactamase										+										Ampicillin resistance <sup>199</sup>		PAA analysis <sup>202</sup>	1995
PFK										+										Activation of PFK, necessary for spore formation		KA, MS, mutagenesis <sup>203-205</sup>	2002
KapB										+										Modulating factor for PFK phosphorylation by Pkn4		Yeast two-hybrid, KA <sup>203</sup>	2005
Pkn4										+										6-phosphofruktokinase (PFK) kinase		KA, PPA <sup>205</sup>	2002
Pkn5										+										Negative regulator for vegetative cell,		KA, PPA <sup>206</sup>	1996
Pkn6										+										Positive regulator for vegetative cell		Isothermal titration calorimetry <sup>207</sup>	2007
Pkn9										+										Necessary for cell growth, spore, KREPS9 proteins		KA, PAA analysis, 2D gel <sup>207</sup>	1997
MrpC										+										Transcription factor; fruiting body development		KA <sup>22,82,83</sup>	2005
Pkn14										+										Regulation of <i>mrpC</i> expression ( <i>c.f. fig. 3</i> )		KA, PPA <sup>22</sup>	2005
<b><i>Pseudomonas aeruginosa</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrates Fha1 and Kinase PpkA																				Protein secretion, virulence		KA, MS, mutagenesis <sup>208,209</sup>	2007
<b><i>Staphylococcus aureus</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrates Kinase PknB										Comment													
PknB										So-called <i>S. aureus</i> PrkC										3D structure <sup>159</sup>		2011	
AFT-2										+										Human transcription factor		Peptide arrays, KA, MS <sup>210</sup>	2010
MgrA										+										Global transcriptional regulator		KA <sup>211</sup>	2008
PurA										+										Purine biosynthesis		KA <sup>212</sup>	2009
SarA										+										Transcription regulator; virulence		KA, PAA analysis <sup>213</sup>	2010
SA0498										+										Ribosomal protein L7/L12; central metabolism		KA, PAA analysis <sup>214</sup>	2007
SA0545										+										Phosphate acetyltransferase; central metabolism			
SA0729										+										Triose isomerase; central metabolism			
SA0731										+										Enolase; central metabolism			
SA0944										+										Pyruvate dehydrogenase; central metabolism			
SA1359										+										Elongation factor P; central metabolism			
SA1499										+										Trigger factor; central metabolism			
SA2340										+										Glyoxalase; central metabolism			
SA2399										+										Fructose biphosphate aldolase; central metabolism			
<b><i>Streptococcus agalactiae</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrates Kinases Stp1										Comment and Reference													
CovR										+										Response regulator; toxin expression		KA <sup>215,216</sup>	2003
DivIA										+										Cell division		KA <sup>217</sup>	2009
PpaC										+										Mn-dependent inorganic pyrophosphatase; virulence		KA, PAA analysis <sup>218</sup>	2003
PurA										+										Purine biosynthesis		KA <sup>219</sup>	2005
<b><i>Streptococcus pneumoniae</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrates Kinases StkP										Comment and Reference													
Spr0334										+										-		<i>In vivo</i> 2D gel, MS, KA <sup>220</sup>	2010
PpaC										+										Mn-dependent inorganic pyrophosphatase; virulence			
DivIVA										+										Cell division			
FtsZ										+										Cell division		KA <sup>221</sup>	2010
RitR										+										Transcriptional regulator; iron transport		KA <sup>222</sup>	2009
GlmM										+										Cell wall		2D gel, KA <sup>223</sup>	2005
<b><i>Streptococcus pyogenes</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrate HLP and Kinase Stk																				Histone-like protein		KA <sup>224</sup>	2006
<b><i>Streptomyces coelicolor</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrate AfsR and Kinase AfsK																				Transcriptional regulator, antibiotic biosynthesis ~PknD with PQQ-containing $\beta$ -propeller structure		KA <sup>68</sup>	1994
<b><i>Yersinia spp.</i></b>										<b>Function</b>										<b>Methodology-References</b>	<b>Year</b>		
Substrate Eukaryotic actin and Kinase YpkA																				Cytoskeleton		KA <sup>225</sup>	2000

KA: *in vitro* kinase assay; MS: mass spectrometry; PAA: phospho-amino acid analysis; 2D gel: Two-dimension electrophoresis

Searching for the origin of Ser/Thr kinases in prokaryote, Han & Zhang<sup>152</sup> proposed that Ser/Thr kinases existed before the divergence between prokaryotes and eukaryotes during evolution, or were vertically but not horizontally transferred into prokaryotes at the early stages of bacterial evolution. These enzymes are now considered ubiquitous in bacteria; though His-kinases of TCS outnumber Ser/Thr kinases in any given bacterial strain<sup>152</sup>. However, it recently became obvious that bacterial proteins phosphorylated on Ser/Thr residues largely outnumber the phospho-His/Arg in TCSs, with over 100 phosphorylation sites detected per bacterial cell (*Tab. 2*). This paradox led to the hypothesis that one Ser/Thr kinase can be used in different metabolism pathways to make a phosphorylation cascade. This theory was indeed proven when Nariya & Inouye<sup>22</sup> in 2005 demonstrated the first functional bacterial Ser/Thr kinase cascade (*Tab. 1 & 2*), (*Fig. 3*).



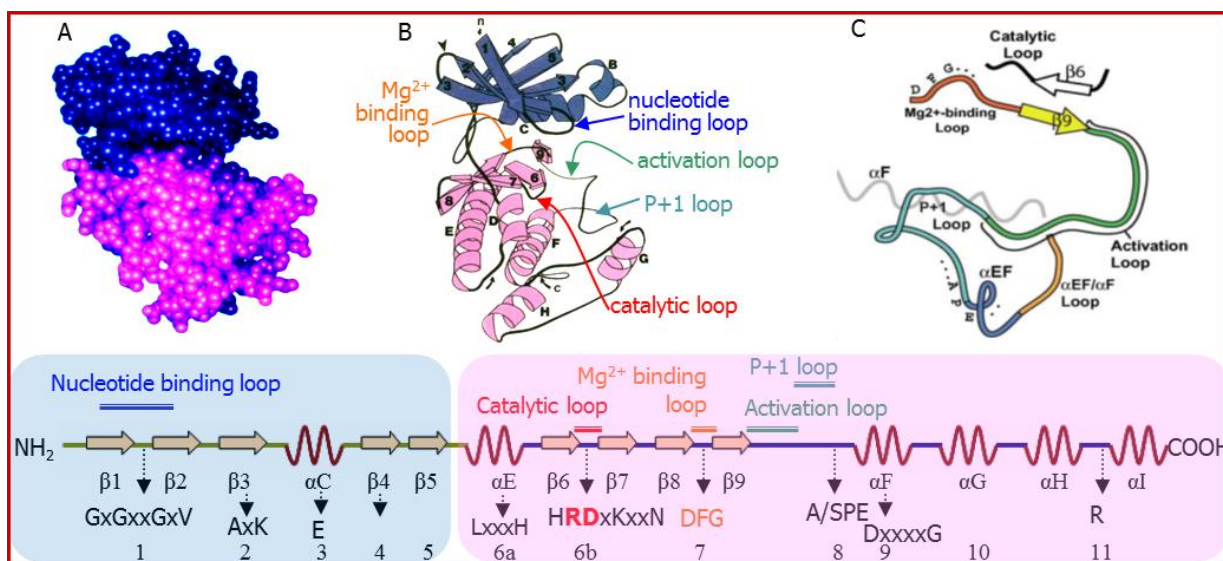


**Figure 3:** Dual regulation of *MrpC* by S/T-kinase cascade and H/D-TCS in *M. xanthus* (reproduced based on <sup>23,154,226</sup>)

The *mrpC* gene is located downstream of the *mrpAB* operon encoding MrpA (His-Sensor Kinase) and MrpB (Asp-Regulator Response) of the TCS. The *mrpC* gene is initially transcribed at a low level by  $\sigma^{54}$ -RNA polymerase in the presence of basal levels of phosphorylated MrpB. Expression of *mrpC* is further amplified by MrpC itself and by proteolytical processing to MrpC2 under the action of LonD protease. However, during vegetative growth, the autophosphorylated Pkn14 is activated by the membrane-associated Pkn8 kinase and therefore phosphorylates MrpC at Thr 21 and/or Thr 22. Phosphorylated MrpC has lower binding affinity to the *mrpC* and *furA* promoters than MrpC. Thus, the Pkn8-Pkn14 kinase cascade prevents sporulation under unfavorable conditions by negatively regulating the DNA-binding activity of MrpC and by preventing the production of MrpC2, which has a higher affinity to the *mrpC* and *furA* promoters <sup>154</sup>.

The similarity between the bacterial protein kinases and their eukaryotic homologues is based on sequence and structural features of their catalytic domain. This catalytic core consists of  $\sim 280$  amino acids in length <sup>151,227</sup> and includes 12 specific conserved subdomains <sup>124</sup> that fold into 2 lobes. The smaller in blue, N-terminal lobe with antiparallel  $\beta$ -sheet architecture (Fig. 4) contains subdomains 1 to 5 and is responsible for nucleotide fixation and orientation <sup>151</sup>. The larger C-terminal lobe in pink, which is dominated by helical structure, is involved in both substrate binding and initiation of phosphotransfer reaction. The catalytic active site between  $\beta_6$  and  $\beta_7$  lying in a deep cleft formed between the two lobes (red arrow), contains conserved subdomains 6b. This loop appears to be the central hub that communicates between different parts of the protein. It not only directs the catalytic event, but also guides the peptide into its proper orientation so that catalysis can occur <sup>228</sup>. The nucleotide binding loop with subdomain 1 (GxGxxGxV motif) forms a deep cleft exposed to the interface between the 2 lobes <sup>229</sup> (blue arrow). The activator segment (green arrows) is composed of Mg<sup>2+</sup> binding loop, activation loop and P+1 loop <sup>230</sup> (subdomains 7, 8, 9 respectively).

Based on available crystal structures, 2 main modes of eSTK activation were generalized; both involve motion of the NH<sub>2</sub>-lobe <sup>229</sup>. In the first case, a eSTK is activated by the (auto)phosphorylation of the activation loop at S/T residue(s). This phosphoresidue in turn interacts with the invariant Arg immediately preceding the catalytic Asp in the catalytic loop of the so-called RD kinases <sup>230-232</sup>. This modification promotes other interactions (which varies from kinase to kinase) in order to create the correct orientation of the activation segment to recognize substrate. In the second mode, a eSTK is activated through binding of a cyclin. This binding promotes rotation of helix  $\alpha C$  and rearrangement of the activation loop <sup>233</sup>. This finally results in bringing the two lobes together in an active conformation <sup>23</sup>. In other words, activation loop phosphorylation and/or  $\alpha C$  rotation following cyclin binding create the substrate binding site <sup>230,232</sup>. Such modifications bring the  $\gamma$ -phosphate of nucleotide (previously stabilized by both Mg<sup>2+</sup> and nucleotide binding loops), the phospho-acceptor S/T residue in the substrate (bound to P+1 loop <sup>23</sup>) and the Asp residue (catalytic loop) closer together <sup>23,231</sup>. The phosphotransfer is ready to occur.



**Figure 4:** Catalytic core of Hanks-type kinases (reproduced based on <sup>227,228,230,234,235</sup>)

However, as nature itself favors diversity, generalization is not always possible. Although no eSTK could be detected in *E. coli* based on consensus motif searching<sup>236,237</sup> (tab. 3), the crystal structure of YihE clearly revealed a typical eSTK fold<sup>101,238</sup>. This protein of unknown function belongs to the AK family (atypical kinases) that shares homology with the catalytic core of eSTKs but does not possess all of the usual kinase motifs<sup>239,240</sup>. Furthermore, not all bacterial S/T kinases are eSTKs (tab. 4). Cross-talk between non-eSTK S/T kinases & His kinases of TCSs also exists and will be described in the following paragraph.

### I.1.3.3. Two-component system-like Ser/Thr/Tyr kinase

All TCS His-kinases are identified by unique signature sequences called H, N, G<sub>1</sub>-F-G<sub>2</sub> boxes<sup>132,241-243</sup>. On the basis of their domain organization, they have been grouped into 2 major classes. EnvZ represents the first class (orange fan, fig. 5A), where the H-box is directly linked to the region of 4 other boxes. The second class is exemplified by CheA with a distinct domain inserted between the H-box and the remaining parts (blue fan, fig. 5A).

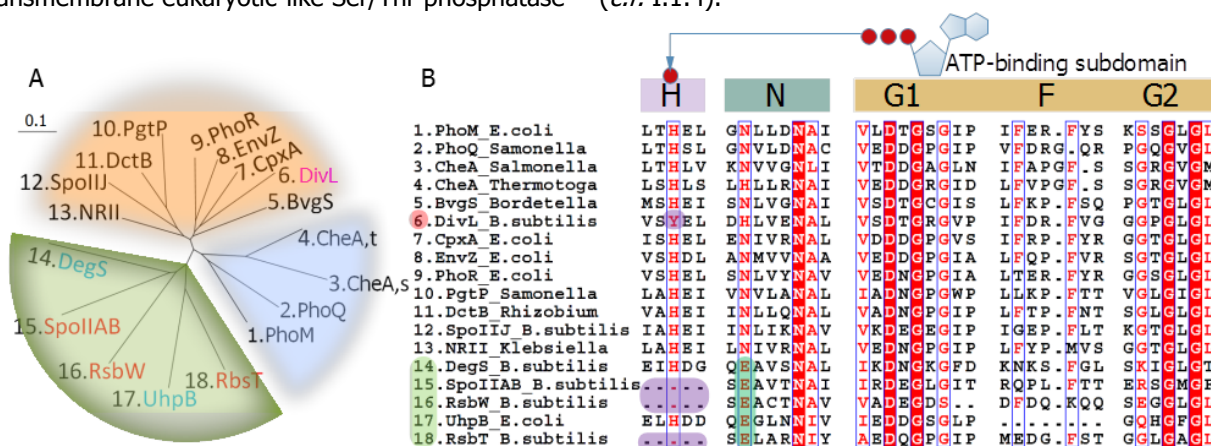
Most TCS His-kinases are transmembrane proteins, presumably activated by extracellular signals via their N-terminal signal-sensing domains protruding from the cell surface. However, little was known about how input signals activate cytosolic His-kinases, e.g. DegS. The TCS DegS/U has been studied for more than 25 years<sup>132,150</sup> and was shown to regulate many of phase transition phenomena<sup>244,245</sup>. In 2007, DegS was found to be phosphorylated on Ser 76 located in its sensing domain by phosphoproteome study of *B. subtilis*<sup>28,94</sup>. In 2010, this Ser-phosphorylation was established as an additional trigger for this TCS. Furthermore, DegS was shown to be phosphorylated by two eSTKs, YbdM and YabT. It was the first example of TCS kinase regulation through its phosphorylation by a eSTK<sup>164</sup>. However, it was not the first reported cross-talk between His-kinases and Ser/Thr as well as Tyr kinases.

In 2001, both eSTK and His-kinase domains were found fused into the single protein HstK in *Cyanobacteria*. Two other similar proteins were also found in the eukaryote *S. pombe* and the Gram-positive bacterium *Rhodococcus*, suggesting that such a coupling mechanism is not a rare event<sup>246</sup>.

In 1999, Wu & al.<sup>75</sup> pointed out a new class of kinase that shows homology to TCS sensory kinases but autophosphorylates on a Tyr residue located in place of the conserved His residue in H-box (fig. 5B, DivL). Upon autophosphorylation on Tyr, the cognate response regulator CtrA is phosphorylated on Asp to probably regulate cell division and differentiation.

In 1993, Min & al.<sup>64</sup> identified the anti- $\sigma^F$ -SpoIIAB from *B. subtilis* which shows some similarity to His-kinases, but is indeed a protein kinase that phosphorylates the anti-anti- $\sigma^F$ -SpoIIAA on a Ser-residue as part of a regulatory circuit that controls the activity of the  $\sigma^F$  in sporulation. SpoIIAB adopts the GHKL superfamily fold of ATPases and His-kinases<sup>247</sup>. Both GHKL (Gyrase, Hsp90, MutL) ATPases family<sup>248</sup> and His-Kinases family, contain the 3 conserved motifs N-G<sub>1</sub>-G<sub>2</sub> (fig. 5B) of the so-called Bergerat ATP-binding fold<sup>239,249</sup>. Although having the conserved catalytic Glutamate in the N-box (one feature of GHKL-ATPases), SpoIIAB however does not possess any ATPase activity. It was supposed that SpoIIAB might be an ancestral protein module of the ATPase/kinase domains of the GHKL superfamily<sup>250</sup>.

Before 2004, SpoIIAB was thought to bear no homology to eSTK. Nevertheless, the crystal structure of the complex SpoIIAB-ATP-SpoIIAA solved by Masdua & al.<sup>251</sup> revealed some critical enzymatic elements comparable with the structure of a eSTK bound to its substrate (PHK-AMPPNP-PS). These similarities were supposed to be due to a convergent evolution mechanism, since they emanate from completely unrelated structural and sequence contexts. Interestingly, although SpoIIAB is not an eSTK, its phosphatase partner SpoIIE is a transmembrane eukaryotic-like Ser/Thr phosphatase<sup>252</sup> (c.f. I.1.4).



**Figure 5: Phylogenetic (A) and Multiple alignment sequence (B) analysis of 18 bacterial "Histidine" kinases** (PhoM-NP\_418816.1, PhoQ-NP\_460200.1, CheA<sub>s</sub>-AEF07797.1, CheA<sub>t</sub>-AAA96387.1, BvgS-AAA22970.1, DivL-AF083422\_1, CpxA-AAN83291.1, EnvZ-AP\_004386.1, PhoR-AAN78987.1, PgtP-CBW18467.1, DctB-CAA77619.1, SpoIIV-AAA22800.1, NRII-ABR79556.1, DegS-NP\_391430.1, SpoIIAB-BAA12654.1, RsbW-NP\_388353.1, UhpB-YP\_859272.1, RsbT-NP\_388350.1) was collected from <http://www.ncbi.nlm.nih.gov/>. The phylogenetic tree and sequence alignment were constructed using the programs ClustalX, ESPript and Treeview.

In the same way, RsbT and RsbW (which belong to the GHKL superfamily) phosphorylate their substrates RbsS and RbsV on S59 and S56 respectively. These kinases are directly involved in partner-switching activity, thus indirectly control the activity of  $\sigma^B$  and the stress response network<sup>253</sup>. Their partners include two serine-phosphorylated substrates and three cytosolic eS/Tphosphatases RsbX, RsbU and RsbP<sup>254</sup> (c.f. I.1.4).

Cross-talks therefore exist between TCS-Histidine kinases and Ser/Thr/Tyr kinases not only at cellular functional level (MrpC in *M. xanthus*, fig. 3 and DegS in *B. subtilis*), but also at sequence (HstK in *Cyanobacteria*<sup>246</sup>, DivL in *B. subtilis*, fig. 5, #6) and structural (SpoIIAB in *B. subtilis*) levels.

#### I.1.3.4. Eukaryotic-like Tyr kinases (eYK)

The overall architecture of the eukaryotic Tyr-kinase domain is similar to that of the eSTKs with a small N-lobe of  $\beta$  sheets and a larger C-lobe of  $\alpha$ -helix (fig. 4). Nucleotide binds in the cleft between the two lobes, and the Tyr-containing substrate binds to the C-lobe. Several residues are highly conserved in all protein kinases, including some Gly in the nucleotide-binding loop, a Lys in  $\beta_3$ , a Glu in  $\alpha_C$ , an Asp and Asn in the catalytic loop, and a DFG motif in the beginning of the activation loop<sup>255</sup>. The most striking indicator of S/T or Y specificity is found in subdomain 6b where the motif DLKPN is a strong indicator of eSTK in place of DLRAAN or DLAARN in eYK. Another motif lying in subdomain 8 is highly conserved among the eYK [P(I/V)KRW(W/M)APE]; with a more limited conservation among the eSTK [G(T/S)xx(Y/F)xAPE]<sup>124</sup>.

While eSTKs are found ubiquitous in bacterial Ser/Thr phosphorylation, Hanks-family seems not to be the preference of bacteria for Tyr phosphorylation<sup>96</sup>. Most of bacterial tyrosine kinases fall into the sole class BY kinases of P-loop motif which will be explored in the next chapter. Only two proteins resembling eYKs, have been identified so far.

Mask from *M. xanthus* is an essential membrane protein which was shown to autophosphorylate on Tyr. The role of Mask remains unclear, however it interacts with the small GTPase MglA and thereby apparently regulate motility and development<sup>256</sup>.

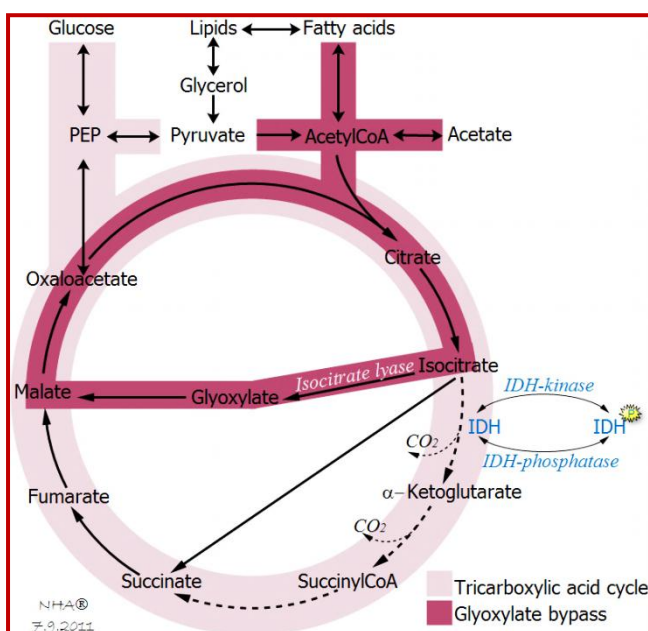
In *P. aeruginosa*, the gene *waaP* belongs to the lipopolysaccharide (LPS) synthesis operon. WaaP possesses not only Tyr-autokinase activity, but also heptose kinase activity allowing it to phosphorylate the first heptose of the LPS core oligosaccharide<sup>257</sup>.

Surprisingly, the tyrosine kinase activity of these enzymes was not observed nor predicted in their homologous proteins in other bacteria (according to homologous search using <http://www.uniprot.org/> or [http://npsa-pbil.ibcp.fr/cgi-bin/npsa\\_automat.pl?page=/NPSA/npsa\\_fasta.html](http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_fasta.html) with *M. xanthus* Mask and *P. aeruginosa* WaaP as queries)

#### I.1.3.5. Isocitrate Dehydrogenase Kinase/Phosphatase (IDHK/P)

While Ser/Thr/Tyr phosphorylation had been extensively characterized in eukaryotes in the 1950's, the first prokaryotic protein substrate to be identified in 1979 was Isocitrate dehydrogenase (IDH) in *E. coli*<sup>14</sup>. This enzyme was found to be phosphorylated on a single serine residue either *in vitro* or *in vivo*<sup>258</sup>.

The IDH phosphorylation cycle controls the flow of Isocitrate through the glyoxylate bypass, a variation of the Krebs' cycle. The presence of this bypass is evident in plants and microorganisms but remains controversial in animals<sup>259,260</sup>.



The glyoxylate bypass centers on the conversion of acetyl-CoA to glyoxylate or succinate directly from isocitrate, thus bypasses the decarboxylation step (causing the loss of entering carbon as CO<sub>2</sub>) in Krebs' cycle<sup>259</sup>. It allows cells to survive on simple carbon sources such as acetate or fatty acids when the preferred carbon source in most organisms, glucose, is not available<sup>261</sup>. During growth on acetate, 75% of IDH is converted to the inactive, phosphorylated form, inhibiting Krebs' cycle and hence forcing isocitrate through the bypass<sup>259</sup>.

The structure of IDH from *E. coli*<sup>262</sup> and *B. subtilis*<sup>262</sup> confirmed that the unique phosphorylatable residue (Ser-113 and Ser-104 respectively) locates in the active site of IDH and forms a hydrogen bond with isocitrate in the active, dephosphorylated enzyme<sup>259</sup>. Phosphorylation of IDH blocks binding of isocitrate by disrupting this bond and by introducing electrostatic repulsion between the phosphate group and isocitrate<sup>263,264</sup>.

**Figure 6:** Role of IDHK/P in Glyoxylate bypass (based on<sup>259,261,265,266</sup>)

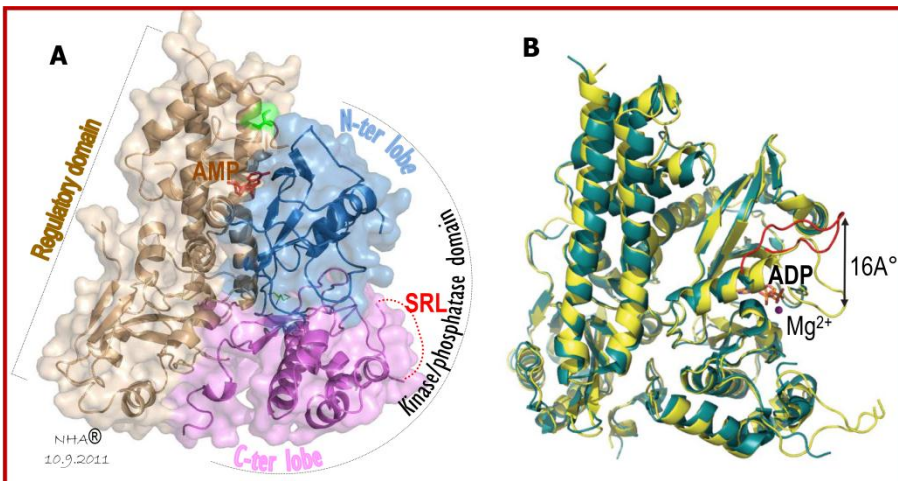


Obviously, phosphorylation of IDH controls the switch between Krebs' and glyoxylate cycle. But what controls the IDH phosphorylation cycle?

-The cycle is catalyzed by a bifunctional kinase/phosphatase IDHK/P<sup>56</sup> encoded by a single gene *aceK*<sup>267,268</sup>. This enzyme also exhibits a strong intrinsic unusual ATPase activity<sup>269,270</sup>. It has been shown that all three activities occur at the same active site<sup>270,271</sup> and a model for catalytic mechanism was proposed<sup>259,270</sup>.

-IDH kinase can use ATP but no other nucleoside triphosphates as a phosphate donor. IDH phosphatase requires a nucleotide (ATP or ADP) for activity. The phosphorylation state of IDH *in vivo* is proposed to be controlled by the levels of isocitrate, PEP, NADPH and the adenine nucleotides<sup>272</sup>.

-The structure of *E. coli* AceK and its complex with IDH solved in 2010<sup>16</sup> revealed a eukaryotic protein-kinase-like domain (*fig. 7*) and a regulatory domain with a novel fold. AMP, as an AceK phosphatase activator and kinase inhibitor<sup>272</sup>, is found to bind in an allosteric site between these two domains (*fig. 7A*). An AMP-mediated conformational change exposes and shields ADP, acting as a switch between AceK kinase and phosphatase activities (*fig. 7B*).



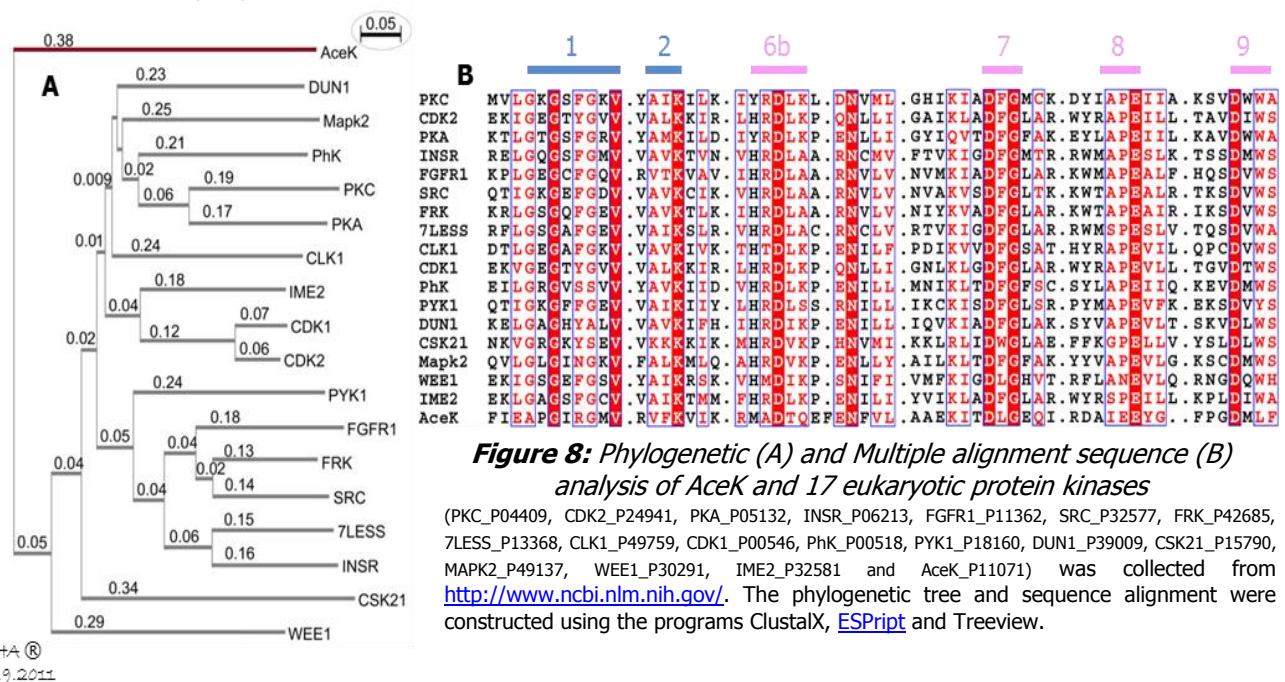
**Figure 7:** Structure of AceK (based on<sup>16</sup>)

A: AceK with AMP between two domains where the kinase/phosphatase domain reveals a eukaryotic kinase fold of two lobes. SRL, the substrate recognition loop binds to the IDH dimer.

B: Switch between kinase & phosphatase activities: AMP-free kinase (marine blue, closed conformation) & AMP-bound phosphatase (yellow, open conformation)

The association of IDH kinase, phosphatase and ATPase on the same polypeptide led to the proposal that *aceK* might have arisen from the imperfect duplication of a primordial kinase gene. Such event of evolution might allow water to gain access to one of the active sites, providing a basis for the phosphatase or ATPase reaction. This suggestion provides a variety of alternative explanations for the absolute requirement of IDH phosphatase reaction for ATP or ADP<sup>56,273</sup>.

In order to clarify if AceK is an evolutionary product of other eukaryotic Ser/Thr kinases, we proceeded to compare its sequence to 17 other eukaryotic-kinase sequences (*fig. 8*). Some similarities do exist at the level of both NH<sub>2</sub>-lobe (blue) and COOH-lobe (pink) (cf. I.1.3.2 for more detail). However, phylogenetic analysis highlights the distance between AceK and other eSTKs. Obviously this enzyme presents a distinct class of Ser kinases harboring slight cross-talks with eSTKs.



**Figure 8:** Phylogenetic (A) and Multiple alignment sequence (B) analysis of AceK and 17 eukaryotic protein kinases

(PKC\_P04409, CDK2\_P24941, PKA\_P05132, INSR\_P06213, FGFR1\_P11362, SRC\_P32577, FRK\_P42685, 7LESS\_P13368, CLK1\_P49759, CDK1\_P00546, PhK\_P00518, PYK1\_P18160, DUN1\_P39009, CSK21\_P15790, MAPK2\_P49137, WEE1\_P30291, IME2\_P32581 and AceK\_P11071) was collected from <http://www.ncbi.nlm.nih.gov/>. The phylogenetic tree and sequence alignment were constructed using the programs ClustalX, ESPript and Treeview.



### I.1.4. Classification of bacterial protein phosphatases

Eukaryotic protein phosphatases are classified into 2 superfamilies subdivided into 6 families<sup>274–276</sup>.

- PSP (*i.e.* **P**hospho **S**er/**T**hr **P**hosphatase) superfamily contains 2 families:
  - i. PPP covers the major sources of protein-Ser/Thr phosphatase activity in mammalian cells
  - ii. PPM consists of metal-dependent Ser/Thr protein phosphatases
- PTP (*i.e.* **P**hospho **T**yr **P**hosphatase) superfamily contains 4 families:
  - iii. cPTP groups conventional Tyr-specific phosphatases,
  - iv. DsPTP family shows dual-specificity Ser/Thr – Tyr protein phosphatase activity
  - v. Cdc25-PTP shows DsPTP activity but has different fold in term of protein structure<sup>275</sup>.
  - vi. LMW-PTP regroups the low molecular weight PTPs that dephosphorylate aryl phosphate esters.

In prokaryotes, the need for dedicated phosphatases was not initially appreciated in the context of TCS and phosphorelay signal transduction, since both phosphohistidine and aspartyl-phosphate residues undergo rapid hydrolysis without phosphatases intervention. In contrast, phosphorylated Ser, Thr, and Tyr residues are not as labile, and therefore cognate phosphatases can be necessary in order to quench signaling cascades<sup>277</sup>. Bacterial phosphatases thus for a long time were restricted to IDHK/P and HPrK/P paradigms.

It was not until 1988, when Cohen & al.<sup>61</sup> encountered an ORF of bacteriophage  $\lambda$  whose predicted protein product PP- $\lambda$  resembled a eukaryotic PPP, that the importance of these bacterial phosphatases were revisited. Shortly thereafter, YopH, a cPTP-like phosphatase encoded by the virulence megaplasmid present within pathogenic strains of *Yersinia* was revealed<sup>63</sup>. Subsequent reports expanded the number of bacterial organisms known to harbor eukaryotic-like protein phosphatases (*Tab. 7*). Today, except Cdc25-PTP family, the 5 other eukaryotic-PTP families are certainly present in bacteria. Remarkably in 2002 Morona & al.<sup>78</sup> for the first time discovered a bacterial protein Tyr-phosphatase among PHP<sup>(\*)</sup> superfamily which is distinct from other PTP families in term of sequence, structure and therefore mechanism<sup>278</sup>. The PHP-PTP family thenceforth became the sixth family of bacterial protein phosphatases and found exclusively in bacteria.

In this section, the distinguishing features of each of these protein phosphatase families will be introduced by briefly reviewing the key attributes at the level of amino acid sequence and/or 3D structure (*Tab. 6*). Next, the physiological and functional properties of established family members will be surveyed (*Tab. 7*).

#### I.1.4.1. PPP family

In the Eukarya, PPP-family covers the major sources of protein-Ser/Thr phosphatase activity. The common catalytic core domain contains  $\approx 280$  amino acids in length that is highly conserved. The sequence signature of the PPPs consists of a trio of short sequence motifs separated by gaps of approximately 25–30 residues:  $\text{GDxHG-x}_{25-30}\text{-GDxxDRG-x}_{25-30}\text{-GNH(D/E)}$ <sup>279, 280</sup>. These eukaryotic phosphatases are metalloenzymes<sup>281–285</sup> that tenaciously bind a pair of closely juxtaposed metal ions, probably  $\text{Fe}^{3+}$  and either  $\text{Zn}^{2+}$  or  $\text{Mn}^{2+}$ , through the amino acid residues within the conserved sequence motifs<sup>281–283</sup>. During catalysis, this bimetallic center activates a water molecule that directly attacks and hydrolyzes the phosphoester bond on phosphoprotein substrates<sup>281,285,286</sup>.

PPP family is divided into 3 classes: PP1, PP2A and PP2B (calcineurin), according to their sensitivities to different inhibitors and their dependence on ions. PP1 and PP2A are not dependent on divalent cations, but PP2B is affected by the presence of  $\text{Ca}^{2+}$ . In 1976 two heat-stable and trypsin-labile phosphorylase phosphatase inhibitors termed inhibitor 1 and 2 were found<sup>287</sup>. PP1 is sensitive to these inhibitors<sup>288–290</sup>, while PP2A and PP2B are resistant<sup>20</sup>. The most widely studied inhibitors of PP1 include: microcystins<sup>292</sup>, okadaic acid<sup>293</sup>, calyculin A<sup>294</sup>, nodularin, tautomycin, fostriecin, cantharidin and belizeanic acid<sup>295</sup>. Since PP1, PP2A, PP2B have highly similar active sites, these toxins also inhibit PP2A and PP2B, but with different affinities. The molecular basis of these differences is not well understood.

In bacteria,  $\approx 8$  PPP phosphatases have thus far been identified and characterized (*Tab. 7*). All of them required an exogenous metal ion such as  $\text{Mn}^{2+}$  for optimal activity and were resistant to potent inhibitors of eukaryotic PPPs such as okadaic acid or microcystin. In marked contrast to their eukaryotic counterparts, the bacterial PPPs all displayed significant activity toward Tyr residues *in vitro* in addition to their expected Ser/Thr phosphatase activity. Pp1-cyano1&2 dephosphorylated His and Lys residues on synthetic amino acid homopolymers<sup>296</sup>, while PrpA&B might possess either His or Asp phosphatase activity<sup>71</sup>.

Bacterial PPPs share sequence homology indicative of an ancestral relationship with the ApaH diadenosine tetraphosphatases<sup>297</sup>, which hydrolyze one of the pyrophosphate bonds to produce either 2 ADP (symmetric) or one AMP and one ATP (asymmetric subgroup). The regions of homology were centered precisely in the areas conserved within the protein phosphatases of the PPP-family, suggesting that ApaH and the PPPs employ a common hydrolytic mechanism. Dixon & al.<sup>281</sup> suggested the five-component signature motif for the metallophosphoesterase superfamily to which bacterial ApaH and PPP family belong:  $[\text{DxHG}]\text{-x}_{\approx 25}\text{-}[\text{GDxxD}]\text{-x}_{\approx 25-30}\text{-}[\text{GH}]\text{-x}_{40-130}\text{-}[\text{Hydrophobic}_2\text{-x}_2\text{-H}]\text{-x}_{25-70}\text{-}[\text{Hydrophobic}_2\text{-x-GH}]$ . This superfamily potentially includes purple acid phosphatase, bacterial exonucleases, as well as some sugar and lipid phosphatases<sup>28,29</sup>.

The identified functions for prokaryotic PPPs include regulation of stress-response<sup>71</sup>, nitrogen fixation<sup>283</sup> and vegetative growth<sup>299</sup>. At least one phosphatase, PrpB from *E. coli*, is implicated in bacterial pathogenesis<sup>300</sup>.

**Table 6:** Classification of bacterial phosphatases (updated from<sup>35</sup>)

Family	Example	Signature sequence motifs	Catalytic mechanism
IDHP/K	AceK	Not determined	phosphotransfer to ADP followed by ATP hydrolysis
HPrK/P	HPrK, PtsK	GxSGxGKSExALELIxRGHxLVADDxVEI, LEIRGLGIIN	unknown, ATP/ADP-independent
PPP-like	PrpC, Stp1	GDxHG-x <sub>25-30</sub> -GDxxDRG-x <sub>25-30</sub> -GNH(D/E)	direct hydrolysis at pSer/pThr
PPM-like	SpoIIE, PstP	(S/T)DGxx(D/E/N), D(D/N)x(T/S)	direct hydrolysis at pSer/pThr
cPTP-like	YopH, YhoP, SptP	D-x <sub>30</sub> -HCx <sub>5</sub> R(S/T)	ping-pong, Cys-P enzyme intermediate at pTyr
LMW-PTP-like	Wzb, PtpA	Cx <sub>5</sub> R-x <sub>85-105</sub> -DP	ping-pong, Cys-P enzyme intermediate at pTyr
Ds-PTP-like	IphP	VxVHCx2Gx2RSx5AY(L/I)M	ping-pong, Cys-P enzyme intermediate at pSer/pThr/pTyr
PHP-like	YwqE, CpsB	HxH, H, H, DxH	unknown
Other	CheY, NRI	(D/E)D-x <sub>40</sub> -(Hydrophobic) <sub>2-4</sub> -D-x <sub>50</sub> -KP	unknown, may stimulate response regulator's autophosphatase activity
	CheZ	AQDxQDLTGQxxKR, QDxxDDLxSLGF	unknown, may activate CheY autophosphatase activity
	Spo0E	Not determined	unknown, may stimulate response regulator's autophosphatase activity
	Spo0L	YxxLxxxR, AE-x <sub>9-11</sub> -E	unknown, may stimulate response regulator's autophosphatase activity

**Table 7:** Bacterial protein phosphatases (updated from<sup>23,35,43,151,300</sup>)

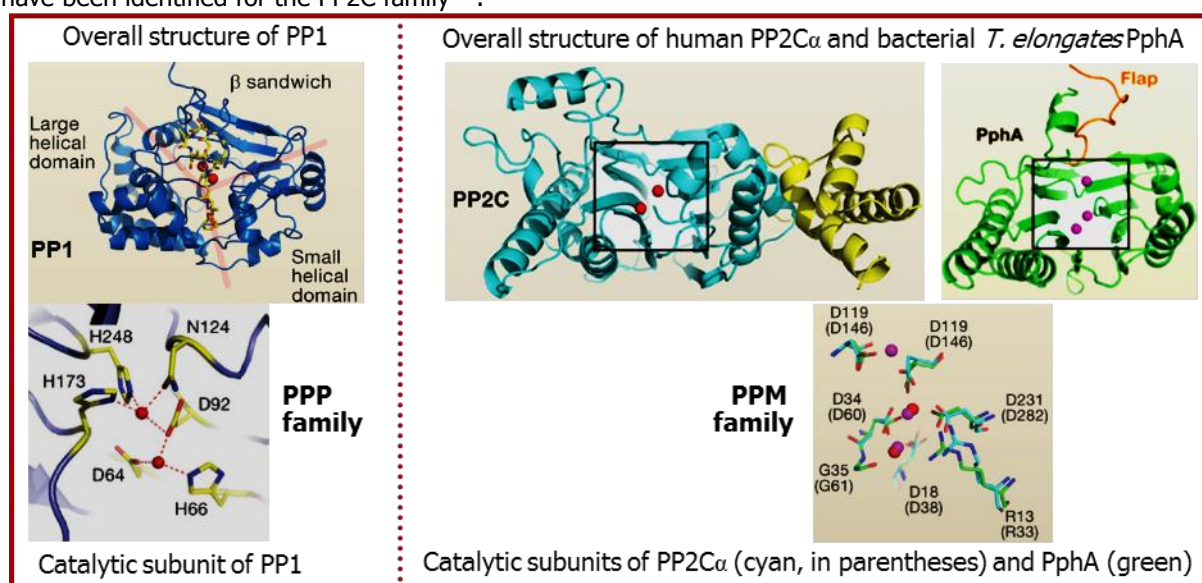
Species	Phosphatase	Class	Partner kinase	Substrates	Function	Methodology	Year
<i>Anabaena sp PCC7120</i>	PrpA	PPP	-	-	Heterocyst formation, nitrogen fixation <sup>291</sup>	<i>In vitro</i> PNPP	1998
<i>Acinetobacter johnsonii</i>	Ptp	LMW-PTP	Ptk	Ptk-pY	Ref <sup>301</sup>	<i>In vitro</i> PNPP & pY peptide	1998
<i>Bacillus anthracis</i>	Stp1	PPM	Stk1	Stk1-P	Growth, virulence <sup>162</sup> , sporulation, germination <sup>302</sup>	<i>In vitro</i> PNPP, pS, pT, pY peptide & Melachite	2010
<i>Bacillus subtilis</i>	PtsK	HPrK/P	-	-	Ref <sup>303</sup>	ORF prediction, <i>In vitro</i> PNPP	
	YjbP (PrpE)	PPP	-	-	Ref <sup>303</sup>	<i>In vitro</i> PNPP	
	PrpC	PPM	PrkC	HPr kinase PrkC-pS/T YezB	Phosphotransferase Germination <sup>157, 304</sup> Stress response <sup>161</sup>	<i>In vitro</i> PNPP, <sup>32</sup> P released <i>In vitro</i> PNPP <i>In vitro</i> <sup>32</sup> P released	2000 2009
				EF-Tu CpgA	GTPase; Translation factor <sup>161</sup> Small ribosome-associated GTPase <sup>161, 305</sup>		2005
				EF-G	GTPase; Translation factor <sup>163</sup>	<i>In vitro</i> PNPP	
	SpoIIE	PPM	SpoIIAB	SpoIIAA-pS	$\sigma^F$ transcription factor <sup>6, 42-45, 309</sup>	Homolog prediction, <i>In vitro</i> PNPP	
	RsbP (YvfP)	PPM	-	RsbV-P	$\sigma^B$ , Energy stress response <sup>254</sup>	<i>In vitro</i> <sup>32</sup> P released	2000
	RsbU	PPM	RsbT/W	RsbV-P	$\sigma^B$ , Environmental stress response <sup>253</sup>	<i>In vitro</i> PNPP	
	RsbX	PPM	RsbT	RsbS-P RsbRA			
	YloO & YvfP	PPM				ORF prediction	
YtrC & YvcJ	cPTP				ORF prediction		
YwqE (PtpZ)	PHP	YwqD	YwqD-pY YwqF-pY SsbA,B TuaD	So-called PtkA UDP-glucuronate biosynthesis <sup>310,311</sup> DNA metabolism, DNA damage response <sup>312</sup>	<i>In vitro</i> PNPP & pY peptide <sup>32</sup> P labelling, 3D analysis <sup>313</sup>	2003 -2005	
YfkJ	LMW-PTP	-	-	$\sigma^B$ , Stress resistance <sup>310,311,314-316</sup>	<i>In vitro</i> PNPP	2005	
YwlE	LMW-PTP	-	McsA, B CtsR	$\sigma^B$ , Ethanol stress resistance <sup>310,311,314-316</sup>	<i>In vitro</i> PNPP, 3D analysis	2005	
<i>Campylobacter jejuni</i>	Cj1258	LMW-PTP	-	-	Ref <sup>317</sup>	<i>In vitro</i> PNPP, 3D analysis	2006
<i>Escherichia coli</i>	AceK	IDHK/P		Idh	controls the switch between Krebs' and glyoxylate cycle <sup>44</sup>		1982
	PrpA	PPP		CpxA-P CpxR-P	Two-component system CpxA/CpxR <sup>71</sup>	<i>In vitro</i> PNPP, <i>In vivo</i> substrates pS, pT, pY peptide	1997
	PrpB						
	Wzb	LMW-PTP	Wzc	Wzc-pY	Capsular polysaccharide colanic acid biosynthesis, resistance to polymyxin <sup>85,278,318-324</sup>	<i>In vitro</i> PNPP, <sup>32</sup> P labelling 3D structure analysis	1999-2010
	Etp		Etk		Stress resistance, $\sigma^E$ , heat-shock response <sup>325</sup>		

<i>Erwinia amylovora</i>	AmsI	LMW-PTP	-	-	Exopolysaccharide synthesis <sup>326</sup>	<i>In vitro</i> PNPP	1997
<i>Klebsiella pneumoniae</i>	Yor5	LMW-PTP	Yco6	Yco6-pY	Capsular polysaccharide biosynthesis <sup>327</sup>	<i>In vitro</i> PNPP & <sup>32</sup> P substrate	2002
<i>Listeria monocytogenes</i>	Stp	PPM	-	EF-Tu MnSOD	Translation factor <sup>328, 329</sup> Superoxide dismutase & Oxidative stress <sup>330</sup>	<i>In vitro</i> & <i>in vivo</i> <sup>32</sup> P labelling	2005 2006
<i>Microcystis aeruginosa</i>	PP1	PPP	-	-	Ref <sup>331, 296</sup>	<i>In vitro</i> PNPP, pS, pT, pY peptide	1997-1999
<i>Mycobacterium tuberculosis</i>	PstP	PPM	PknB	PknA-P PknB-P PBPA	Cell division <sup>332</sup> Cell growth <sup>333</sup> Pencillin binding & cell division <sup>80, 182</sup>	<i>In vitro</i> PNPP Mass Spectrometry 3D structure analysis <sup>80</sup>	2003-2006
	PtpA PtpB	LMW-PTP cPTP	- -	- -	Ref <sup>334</sup>	<i>In vitro</i> <sup>32</sup> P labelling MBP 3D structure analysis <sup>335,336</sup>	2000 2005
	MspP	PPM	-	-	Ref <sup>337</sup>	<i>In vitro</i> PNPP & 3D structure <sup>337</sup>	2007
<i>Mycoplasma pneumoniae</i>	PrpC	PPM	-	HPr	Phosphotransferase system <sup>338</sup>	<i>In vitro</i> PNPP	2006
<i>Myxococcus xanthus</i>	Pph1	PPM	Pkn5	Pkn5	Vegetal growth & development <sup>339</sup>	Yeast two-hybrid & PNPP	2001
<i>Pseudomonas aeruginosa</i>	Stp1	PPM	-	-	Ref <sup>340</sup>	<i>In vitro</i> PNPP, <sup>32</sup> P labelling MBP	1999
<i>Nostoc commun</i>	IphP	DsPTP	-	-	Ref <sup>67, 341</sup>	<i>In vitro</i> <sup>32</sup> P labelling peptides	1993
<i>Salmonella Typhi</i>	PrpZ	PPM	PrkY	-	Resistance to H <sub>2</sub> O <sub>2</sub> & HOCl stress <sup>342</sup> Pathogenesis <sup>343, 344</sup>	<i>In vitro</i> PNPP <i>In vitro</i> <sup>32</sup> P labelling MBP	2005 2009
<i>Samonella typhimurium</i>	SptP	cPTP	-	-	Disrupt the host cell's actin cytoskeleton <sup>345-347</sup>	<i>In vitro</i> peptide	1996
	PrpA PrpB	PPP	-	-	Ref <sup>348</sup>	3D structure analysis <sup>349</sup> <i>In vitro</i> PNPP & <sup>32</sup> P labeling	2003 2001
	Stp1 PtpA & PtpB	PPM LMW-PTP	Stk1 -	Stk1-P -	Exotoxin hemolysin expression, virulence <sup>350,351</sup> Ref <sup>352</sup>	<i>In vitro</i> PNPP <i>In vitro</i> PNPP	2001 2002
<i>Streptococcus agalactiae</i>	Stp1	PPM	Stk1	PpaC CovR PurA	Inorganic phosphatase, cell segregation <sup>215</sup> TCS-mediated expression of cytotoxins. <sup>353</sup> Purine biosynthesis <sup>219</sup>	<i>In vitro</i> PNPP, 3D structure analysis <sup>354</sup> <i>In vitro</i> <sup>32</sup> P labeling	2003-2007
<i>Streptococcus pneumoniae</i>	PhpP	PPM	StkP	StkP-P 5 proteins GlmM RitR	Competence, virulence, transcription <sup>355-358</sup> Glycolysis, pentose metabolism, transcription <sup>356</sup> Cell wall biosynthesis <sup>356</sup>	<i>In vitro</i> <sup>32</sup> P labeling <i>In vivo</i> <sup>32</sup> P labelling & gel 2D <i>In vivo</i> & <i>In vitro</i> <sup>32</sup> P labeling	2004-2005
	CpsB	PHP	-	CpsD-P	Two-component response regulator <sup>222</sup> Encapsulation, virulence <sup>7,53,65,107,108</sup>	<i>In vitro</i> <sup>32</sup> P labeling <i>In vitro</i> pY peptide, 3D structure	2009 2000
	Stp	PPM	Stk	HLP Stk-P	Growth, morphology, cell division/septation, surface protein, virulence factor <sup>224</sup>	Immunoprecipitation <i>In vitro</i> PNPP & <sup>32</sup> P labeling	2006
<i>Streptomyces coelicolor A3(2)</i>	SppA 49 proteins	PPP PPMs	-	-	growth; spores & hyphae formation <sup>70, 299</sup> Ref <sup>361</sup>	<i>In vitro</i> PNPP ORF prediction	2000 2004
	PtpA	LMW-PTP	-	-	Ref <sup>70</sup>	<i>In vitro</i> PNPP & pY peptide	1996
<i>S. avermitilis</i>	48 proteins	PPMs	-	-	Ref <sup>361</sup>	ORF prediction	2004
<i>Synechocystis sp.</i>	Slr1860/IcfG	PPM	Slr1861	Slr1856	carbon metabolism <sup>362</sup>	<i>In vitro</i> <sup>32</sup> P labelling	1999
	PphA	PPM	-	PII	Nitrogen, carbon and redox-signals <sup>363-366</sup>	<sup>32</sup> P labelling & Native gel 1D	2001
	Ptp	LMW-PTP	-	-	Regulation of photosynthesis <sup>367</sup>	3D tructure analysis <sup>368</sup>	2011
<i>Yesinia spp.</i>	YopH	cPTP	FAK	Cas, FYB Paxillin FAK	Virulence, blocking phagocytosis <sup>369,370</sup> Focal adhesions <sup>369-373</sup> Focal adhesion kinase	<i>In vivo</i> & <i>In vitro</i> <sup>32</sup> P labeling 3D structure <sup>374-376</sup>	1990 2004

### I.1.4.2. PPM Family

In eukaryotes, the principal members of the PPM-family are PP2C and pyruvate dehydrogenase phosphatase. Well before their respective signature motifs were known, the PPMs were distinguished from the PPPs by the former's requirement for an exogenous metal ion, usually  $Mg^{2+}$ , to support catalytic activity<sup>377</sup>. Lately,  $Fe^{2+}$  was reported to be far superior to  $Mg^{2+}$  as a cofactor *in vitro*<sup>378</sup>. At the primary sequence level, eukaryotic PPMs possess an  $\approx 290$  residue catalytic domain containing 11 conserved motifs<sup>151,340,379</sup>. The two longest motifs are numbers 8, [S/T]-DGxx[D/E/N] and 11, D[D/N]x[T/S]. Although there is no sequence homology between PPPs and PPMs, X-ray crystallographic analysis has revealed a striking degree of similarity between their active sites, suggestive of convergence to a common catalytic mechanism<sup>378,380</sup>. The only difference consists of ligand and substrate binding features. In PPP enzymes, the active-site-ligands are stabilized by His, Asp and Asn side chains<sup>381</sup>, whereas in PPMs, Asp side chains and Gly backbone carbonyl coordinate the metal ions<sup>354,382</sup>. In the PPP family, the substrate phosphoryl group is bound directly to the two metal ions via its oxygen residues, whereas PPM family enzymes bind the substrate indirectly, via hydrogen-bonding interactions between the phosphoryl group and water molecules liganded to the metal ions<sup>276,382,383</sup>.

All eukaryotic PPMs isolated to date have proven to be monomeric proteins that generally ranged from 31 to more than 150 kDa in size, except for pyruvate dehydrogenase phosphatase which is a heterodimer with a catalytic subunit (PDPc) and a regulatory subunit (PDPp)<sup>384-387</sup>. No dedicated regulatory or targeting subunits have been identified for the PP2C family<sup>382</sup>.



**Figure 9:** Structural comparison of PPP and PPM families (updated from <sup>380</sup>)

In bacteria, the PPMs represent the most prolific and best studied of the protein-Ser/Thr phosphatases. To date,  $\approx 19$  bacterial PPMs have been biochemically characterized (*Tab 7*). They are divided into 2 small groups based on the presence of 5a/5b sequence motif<sup>151,300,368,379</sup>. Except the trimer phosphatase PphA from *Synechocystis*<sup>363-366</sup>, the rest reveals monomeric proteins. The largest bacterial PPM, SpoIIE has a size of 91 kDa due to the presence of a large N-terminal membrane anchor domain with  $\approx 10$  membrane-spanning segments<sup>69</sup>. The N-terminal portion of RsbP contains a predicted PAS domain<sup>254</sup>, an internal energy sensor (e.g.  $O_2$ , redox potential or light<sup>388</sup>). Slr1860/IcfG of 72 kDa also contained considerable extra-sequence whose functional role are still unclear<sup>362,389</sup>.

In 2004, Pullen & al.<sup>80</sup> reported the first structure of a bacterial PPM phosphatase domain of PstP from *M. tuberculosis* and the second altogether, after that of human PP2C $\alpha$ <sup>382</sup>. Despite their low sequence identity  $\approx 17\%$ , their core structures are remarkably similar, including the two central five-stranded  $\beta$  sheets flanked by  $\alpha$  helices as well as the two-metal center and their direct ligands at the active site. However, the active site of PstP contains 3 metal ions, rather than 2 as in PP2C $\alpha$ . The third metal binding site seems to be conserved among bacteria<sup>34,58,70,95</sup> and has putative role in creating an extended substrate binding site for diphosphorylated substrates, or in the mobility of the previously unrecognized flap subdomain. This mobile flap region may facilitate substrate binding or turnover, and may introduce specificity to the dephosphorylation of substrates.

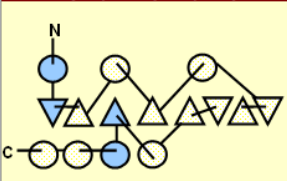
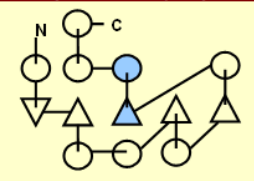
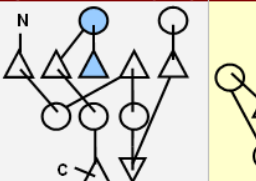
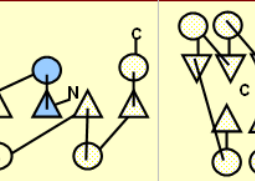
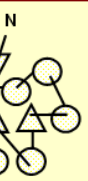
In contrast to PPPs, most prokaryotic PPMs have their physiological substrates determined (*tab. 8*). Their proposed functions cover different bacterial processes, although the molecular mechanisms are largely unknown. They associated with a complex multistage developmental cycle (spore formation, development of fruiting bodies, vegetative growth, or cell segregation, i.e. Stp, PstP, Pph1), with stress responses (transcription factor, competence, i.e. *Bacillus* PPMs, PrpZ, PhpP), with different pathways of metabolisms (carbon, nitrogen, redox, purine, i.e. IcfG, PphA), or even with virulence and pathogenesis (Stp, PphP).

### I.1.4.3. Phospho Tyr Phosphatase superfamily (PTPs)

In eukaryote, there are four topologically distinct PTP families (Tab. 8, the four first columns) that share a common active-site motif [ $Cx_5R(S/T)$ ] and a common topology of  $\alpha/\beta$  fold (all  $\beta$  sheets surrounded by  $\alpha$  helices). One exception to this general rule is Cdc25, which lacks the S/T residue and possesses irregular  $\beta$  sheets. The phosphate-binding loop (also referred to as the PTP signature motif) which resides between the  $\beta$  strand (starting with C) and the  $\alpha$  helix (beginning with R(S/T)) is critical for enzyme activity<sup>276</sup>. Another highly conserved R residue located on a separate loop (acid loop), approximately 30-40 residues away from the active-site, is also required for catalysis<sup>391-393</sup>. One striking difference between the PTPs and the Ds-PTPs resides in the deeper active-site pocket of the former<sup>381, 394</sup>. This difference might contribute to the specificity of PTPs toward phospho-Tyr residues, since the depth of the pocket roughly corresponds to the length of the phosphorylated side chain. In the same way, the active-site of PTEN lipid phosphatase is deeper and wider than that of Ds-PTPs<sup>276</sup>.

All Cys-based PTPs utilize the same catalytic mechanism during which the phosphate on the substrate is first transferred to the Cys residue in the signature motif before being hydrolyzed by water to release the phosphate anion. Thus, most of PTPs are metal-independent<sup>300,35,395</sup>. Bacteria do not have Cdc25-PTPs but possess instead a non Cys-based PHP-PTP family, which function with a  $Mn^{2+}$ -dependent mechanism.

**Table 8:** Topological comparison of 5 subfamilies of PTPs (updated from<sup>276, 396, 397, 335</sup>)

c-PTP (YopH, <i>1ytw.pdb</i> )	Ds-PTP (VHR, <i>1vhr.pdb</i> )	Cdc25 (human, <i>1cwt.pdb</i> )	LMW-PTP (bovin, <i>1pnt.pdb</i> )	PHP-PTP
				
D-X <sub>2-30</sub> -HCX <sub>5</sub> R(S/T)	VxVHCX <sub>2</sub> GX <sub>2</sub> RSX <sub>5</sub> AY(L/I)M	D-X <sub>2-45</sub> -CX <sub>5</sub> R	CX <sub>5</sub> R-X <sub>85-105</sub> -DP	HxH, H, H, DxH

Till 2011, a total of 18 bacterial PTPs have been characterized in detail, including 1 DsPTP, 3 cPTPs, 2 PHP-PTPs & 13 LMW-PTPs (Tab. 7). While few in number, these enzymes embody an intriguing set of properties.

**IphP** from cyanobacterium, *N. commune* was the first bacterial PTP and also the first bacterial DsPTP discovered<sup>67</sup>, in 1993. IphP appears to be a secreted protein that displayed phosphomonoesterase activity toward a broad range of exogenous peptides, proteins, as well as pyrophosphatase activity toward ADP and ATP<sup>341</sup>. Thus, it might function either as dedicated protein phosphatases or as nonspecific phosphate in the mold of the acid and alkaline phosphatases<sup>45</sup>. The physiological roles of IphP remain uncertain<sup>35</sup>.

**YopH** from *Yersinia* was the first cPTP discovered and characterized in bacteria<sup>63</sup> (Tab. 1). This molecular missile is secreted from the bacterium to assault its eukaryotic host where it attacks Tyr-phosphorylated proteins localized to focal adhesions (Tab. 7), thereby disabling host defenses. Besides the C-terminal catalytic domain, the protein contains also the N-terminal sequences involved in substrate-binding, chaperon SycH-binding<sup>398</sup>, secretion from the bacterium and translocation into mammalian cells<sup>372</sup>. *yopH* gene however is not essential for the bacterial growth<sup>399</sup> and is on the same virulence plasmid containing a eukaryotic kinase gene<sup>400</sup>. *yopH* therefore might have been acquired from a eukaryotic host via a horizontal gene transfer<sup>63</sup> and YopH does not act on endogenous bacterial protein<sup>399</sup>.

Although the first bacterial phosphatase activity was reported very early form 1981 in *S. typhimurium*<sup>55</sup> (Tab. 2), it was not until 1996 that **StpP**, the first *Salmonella* phosphatase was characterized<sup>347</sup>. Like YopH, StpP targets proteins within a eukaryotic host cell, where it disrupts the actin cytoskeleton<sup>345</sup>. But in contrast to the potentially mobile plasmid-encoded *yopH*, the gene for StpP resides within the chromosomal DNA of *S. typhimurium*<sup>347</sup>. The C-terminal of StpP is homologous to YopH and the N-terminal is homologous to YopE cytotoxin of *Yersinia*<sup>346</sup> which is involved in disruption of the host cytoskeleton<sup>401</sup>. StpP has cPTP activity and also GTPase-activating protein activity towards two GTPases involved in cytoskeletal dynamics<sup>402</sup>.

**PtpB** from *M. tuberculosis* is another example of a secreted bacterial cPTP. Along with its companion secreted LMW-PTP, PtpA, these 2 PTPs likely target components of host signaling pathways, since no Tyr-kinase was found in the genome of *Mtb*<sup>118</sup>. PtpB exhibited cPTP activity both in term of sequence and biochemical analysis<sup>334</sup>. But one of the over two dozen PTP structures in the PDB nor the structure of the PTEN show close structural homology to PtpB. Phylogenetic analysis suggests that PtpB may represent a distinct branch that shares features of cPTPs as well as DsPTPs<sup>335</sup>.

The first LMW-PTPs were isolated from *A. johnsonii* (**Ptp**)<sup>301</sup>, *E. amylovora* (**AmsI**)<sup>326</sup> and *E. coli* (**Wzb**)<sup>85,278,318-324</sup>. It is now clear that bacterial LMW-PTPs are ubiquitous regulators of Tyr phosphorylation. Despite their abundance, the physiological functions of LMW-PTPs in bacteria remain largely unknown. Several studies demonstrated a role of LMW-PTPs in CPS/EPS synthesis and export. AmsI is located within the *ams* operon

which controls EPS synthesis<sup>326</sup>; *A. johnsonii* Ptp can dephosphorylate the transmembrane autokinase Ptk, which regulates colanic acid synthesis; and both *E. coli* Wzb and *K. pneumoniae* Yor5<sup>327</sup> could regulate CPS production through dephosphorylation of Tyr-kinases Wzc and Yco6 respectively. In other studies, it was concluded that the **Etp** from *E. coli* regulates bacterial resistance to heat shock by preventing the Etk-mediated phosphorylation of the RNA polymerase  $\sigma^{32}$  subunit and the anti- $\sigma^E$  factor RseA<sup>325</sup>. Similarly, **YfkJ** and **Ywie** seem to be involved in *B. subtilis* resistance to ethanol stress<sup>314</sup>. Overexpression of the gene for **PtpA** in *S. coelicolor*<sup>70, 403</sup> led to overproduction of secondary metabolites such as undecylprodigiosin and A-factor. Recently in 2011, *Synechocystis* **Ptp** was reported to be involved in the regulation of photosynthesis through the dephosphorylation of two endogenous substrates, Psad (photo-system I subunit II) and CpcD (phycocyanin rod linker protein)<sup>367</sup>.

**CpsB** from *S. pneumoniae*<sup>78,300</sup> was the first bacterial PHP-PTP discovered (Tab. 2). Being originally classified as PHP<sup>300</sup>, it shows no sequence similarity to PTPs. However biochemical studies confirmed its PTP activity toward CpsD-pY in a Mn<sup>2+</sup> dependent way<sup>78,359</sup>. The *cpsB* gene belongs to the *cps* locus involved in CPS production, along with *cpsA*, *cpsC*, *cpsD* to form the 4 most conserved *cps* genes. CpsD is a Tyr-kinase that requires CpsC to be functional<sup>78</sup>. The complex CpsC-CpsD shows high sequence similarity to the C-terminal and N-terminal of the Tyr-kinase Wzc from *E. coli*<sup>78, 278</sup>. Wzb is also dephosphorylated by its own cognate phosphatase LMW-PTP Wzb<sup>404</sup>. Despite the functional parallels, CpsB and Wzb share no sequence homology, neither structural feature<sup>278</sup>, suggesting that they must operate by very different mechanisms. Inactivation of Wzc, or Wzb, or CpsB impairs CPS production. Mutation of the ATP-binding motif of CpsD results in reduction of CPS biosynthesis, while phosphorylated-site-pY-mutation increases encapsulation, demonstrating negatively that Tyr-phosphorylation of CpsD regulates CPS biosynthesis<sup>359</sup>.

**YwqE** (so-called PtpZ) is the first PTP isolated from *B. subtilis*, and the second PHP-PTP that has been discovered so far. It is located in the same *cps*-like operon with YwqC (so-called TmkA transmembrane modulator), YwqD (so-called Tyr-kinase PtkA), and the UDP-glucose dehydrogenase YwqF (so-called Udg, which catalyze the formation of precursors for acidic polysaccharide biosynthesis)<sup>51,52</sup>. YwqE can dephosphorylate YwqD and its substrates, including both SsbA, SsbB (single-strand DNA-binding proteins, also called YwqH)<sup>312</sup> and YwqF. The phosphorylation state and enzymatic activation of YwqF is controlled by YwqD/YwqE<sup>311</sup>. These two later are also involved in bacterial DNA metabolism and DNA damage response through the phosphorylation system of SSBs<sup>312, 405</sup>. Additional targets of the bacterial phosphorylation system such as single-stranded DNA exonuclease YorK and aspartate semi-aldehyde dehydrogenase Asd have been identified in *B. subtilis*. It was reported that the majority of these proteins could be phosphorylated and activated by YwqD<sup>161,162</sup>. YwqE therefore is involved in polysaccharide biosynthesis, bacterial DNA metabolism, and DNA damage response in *B. subtilis*.

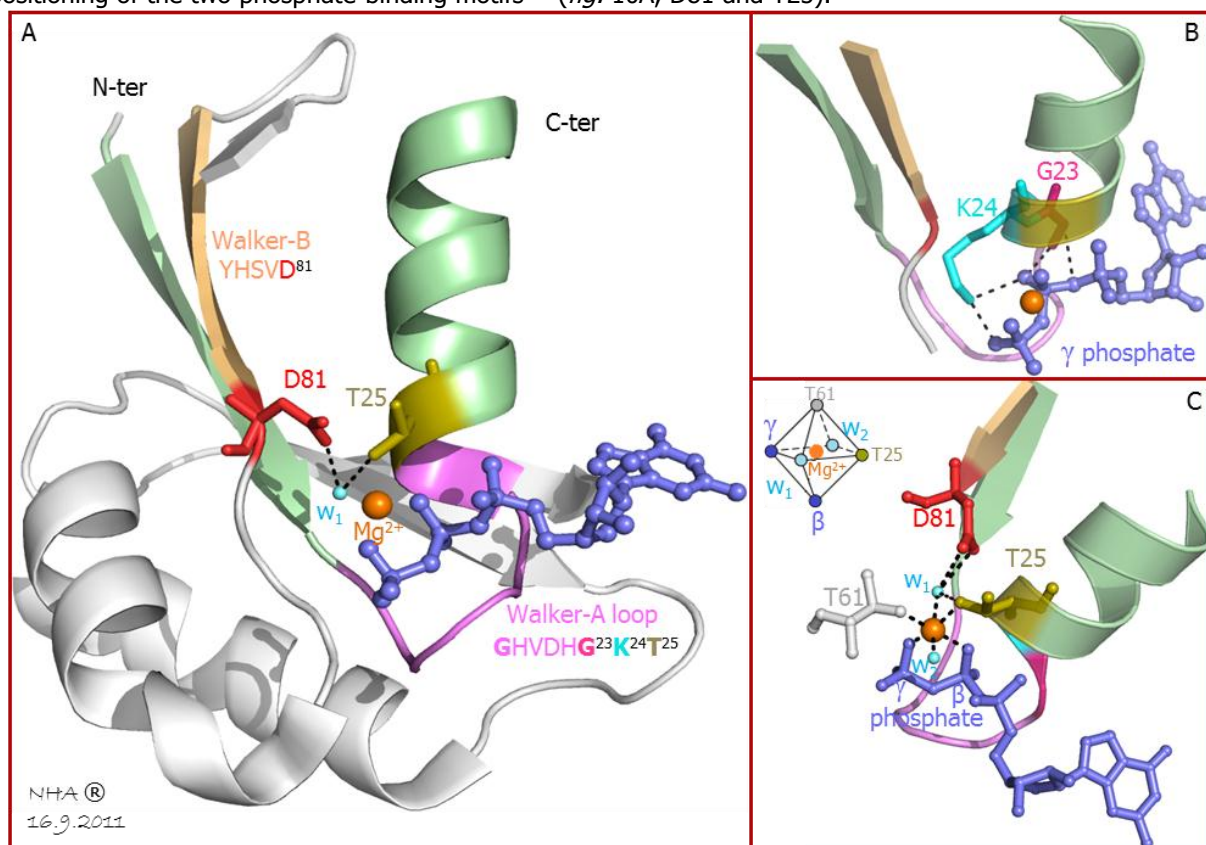


## I.2. Phosphorylation in prokaryotes by Walker-motif-protein kinases

### I.2.1. Overview of Walker motifs and Walker motif-protein kinases

We have listed all bacterial protein kinases known-to-date, whose sequences do not contain the motif  $Gx_4GKT[S]$  (*cf. tab. 3*). This motif was first recognized in 1982 by Walker & al.<sup>76</sup> as a common nucleotide binding fold in the  $\alpha$ - and  $\beta$ -subunits of F1-ATPase, myosin and other ATP-requiring enzymes. Since then, this sequence has been found in many proteins that bind nucleotides and thereby gained predictive value for nucleotide binding site in proteins. However, it is noteworthy that i) not every nucleotide-binding protein needs this motif, *e.g.* all classes of kinases described in previous chapter; ii) not every protein containing such a motif are nucleotide-binding proteins, as proposed by Ramakrishnan & al.<sup>408</sup> in 2002. Of the Walker-motif proteins, only those in which the motif folds in a distinctive loop are nucleotide-binding proteins.

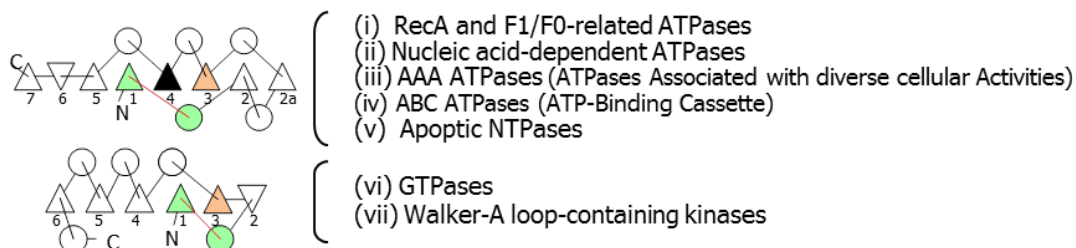
This loop is characterized by a  $\beta$ -strand at the N-ter and an  $\alpha$ -helix at the C-ter<sup>408</sup>, resulting in the motif  $\beta$ -W- $\alpha$  (*fig. 10A*, pink loop). The conserved lysine of the motif forms hydrogen bond(s) with the carbonyls of the main chain to stabilize the loop. Together with the preceding glycine, they interact with the  $\beta$  and  $\gamma$  phosphates of the nucleotide, allowing neutralizing the negative charges of the phosphate groups (*fig. 10B*, G23 and K24). Walker & al. called  $Gx_4GKT[S]$  motif-A and also discovered another motif more variable, which is composed of a conserved aspartate at the C-ter of a hydrophobic strand, hhhhD, where h is a hydrophobic residue. This latter, so-called motif-B, provides a water-bridge for the octahedral coordination of a  $Mg^{2+}$  cation<sup>409,410</sup>, which, in turn, is coordinated to the  $\beta$  and  $\gamma$  phosphate groups of the nucleotide (*fig. 10C*). Furthermore, a hydrogen bond between the Walker-B aspartate and the threonine/serine of Walker-A loop secures the proper relative positioning of the two phosphate-binding motifs<sup>410</sup> (*fig. 10A*, D81 and T25).



**Figure 10:** Walker-A loop and Walker-B motif in nucleotide-binding proteins

Illustration with *Thermus Thermophilus* elongation factor EF-Tu in complex with GTP analogue GPPNHP (*1exm.pdb*)

Leipe & al.<sup>409,410</sup> proposed at least 7 major families inside the nucleotide-binding protein superfamily. They are then structurally regrouped into 2 different folds, based on the presence, or not, of an additional strand (black triangle) inserted between the Walker-A loop-binding strand (green) and the Walker-B strand (wheat).



Inside the second group, the Walker-A kinases are distinguished from the GTPases firstly by the presence of the LID module, which is located between strands 4 and 5; and secondly by the absence of guanine-binding motif [N/T]xxD at the end of strand 5, as well as a conserved glycine associated with the Walker-B motif hhhhDxxG. The LID module is variable but typically composes of Rx<sub>2-3</sub>R, where the first arginine is sometimes replaced by e.g. lysine, tyrosine, histidine, or serine, which could either interact with the adenine moiety of ATP or ADP to hold the base in place<sup>410</sup>. The second arginine, by interacting with the  $\gamma$  phosphate of ATP hence neutralizing the negative charge on the  $\beta$ - $\gamma$  bridge oxygen atom, is believed to stabilize the transition state of phosphorylation.

Here, we analyzed the Walker-A loop-containing kinases. Kinases are ubiquitous enzymes that transfer the  $\gamma$  phosphate of ATP to a wide range of substrates, ranging from nucleotides and other small molecules to nucleic acids and proteins. We are interested in bacterial protein kinases and hereafter will focus on bacterial Walker-A-loop containing protein kinases. So far, only two such systems have been reported in bacteria, namely Histidine Protein Kinases/Phosphorylases (HPrK/P), and Bacterial Tyrosine kinases (BY kinases).

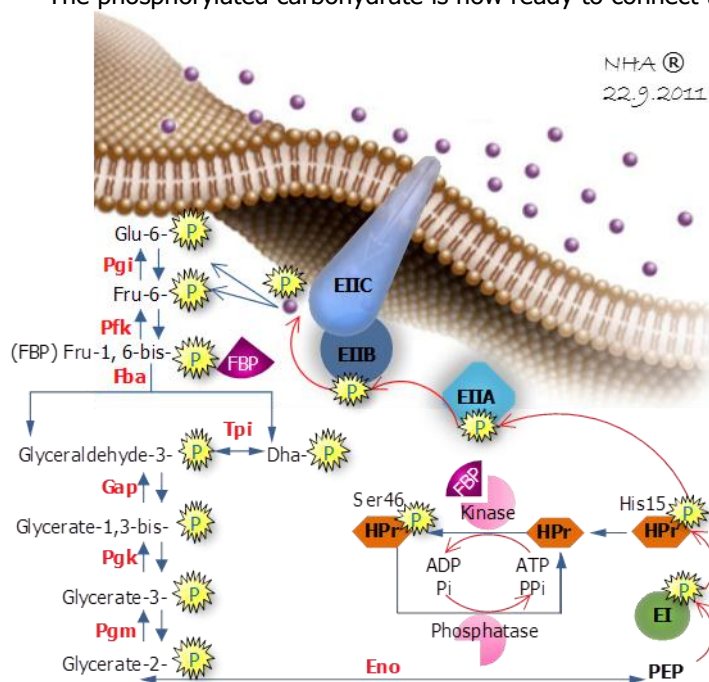
### I.2.2. HPrK/P

In the early 1940s, Jacques Monod observed the biphasic growth (i.e. diauxie phenomenon), when *B. subtilis* was cultivated in a medium containing sucrose and dextran. The cells first utilized sucrose then stopped growing for a certain period (lag/adaptation phase) before resumed growth by utilizing dextran. In subsequent years, it was found that preferred sugars such as glucose, fructose, or sucrose, as long as they are present in sufficient amounts in the growth medium, repress the synthesis of the enzymes necessary for the transport and metabolism of less favorable carbon sources (i.e. CCR-carbon catabolite repression phenomenon). When the preferred carbon source is exhausted, bacteria first need time (lag phase) to synthesize the previously mentioned enzymes before they can resume growth. Interestingly, the bacterial PEP:PTS, which catalyzes the uptake and phosphorylation of numerous carbohydrates, appeared to play a major role in bacterial CCR.

...what is PEP:PTS?...

The PTS was first discovered in *E. coli* by Kundig & al.<sup>411</sup> in 1967 as a system that uses PEP (but not ATP) to phosphorylate a number of hexoses, including glucose, mannose, etc... Subsequently, it was recognized that the PTS is in fact a transport system that catalyzes the uptake of numerous carbohydrates and phosphorylates them concomitantly during transport.

Each carbohydrate PTS is comprised of two cytoplasmic components (i.e. EI-Enzyme I & HPr) and one transmembrane complex (i.e. EII-Enzyme II). The latter determines the carbohydrate specificity and is responsible for the transport of carbohydrate across the bacterial membrane as well as its phosphorylation. The phosphorylated carbohydrate is now ready to connect the PEP/EI/HPr phosphoryl transfer pathway.



EII complex can consist of up to four separate proteins covering an integral membrane domain and cytoplasmic domains. The glucose-specific EII complex of enteric bacteria consists of two distinct proteins, the cytoplasmic protein EIIA<sup>Glc</sup> (encoded by the *crr* gene) and the membrane-associated protein EIIB<sup>Glc</sup> (encoded by the *ptsG* gene). The phosphoryl group is first transferred from  $P_i$ -His-HPr to EIIA, then from  $P_i$ -His-EIIA to EIIB and finally from  $P_i$ -His/Cys-EIIB to the carbohydrate previously transported the membrane towards the cytoplasm by the EIIC domain (fig.11).

EI (*ptsI* gene<sup>412</sup>) is autophosphorylated on a His-residue which is located on the N-ter of the protein. While the N-ter contains the HPr binding site, the C-ter contains the PEP binding site and is necessary for dimerization. It appears that the phosphorylation does not drastically change the conformation of the N-ter domain per se, but the PEP binding provokes structural changes and hence facilitates phosphotransfer.

**Figure 11:** Carbohydrate transport and phosphorylation by the PTS and their coupling to glycolysis

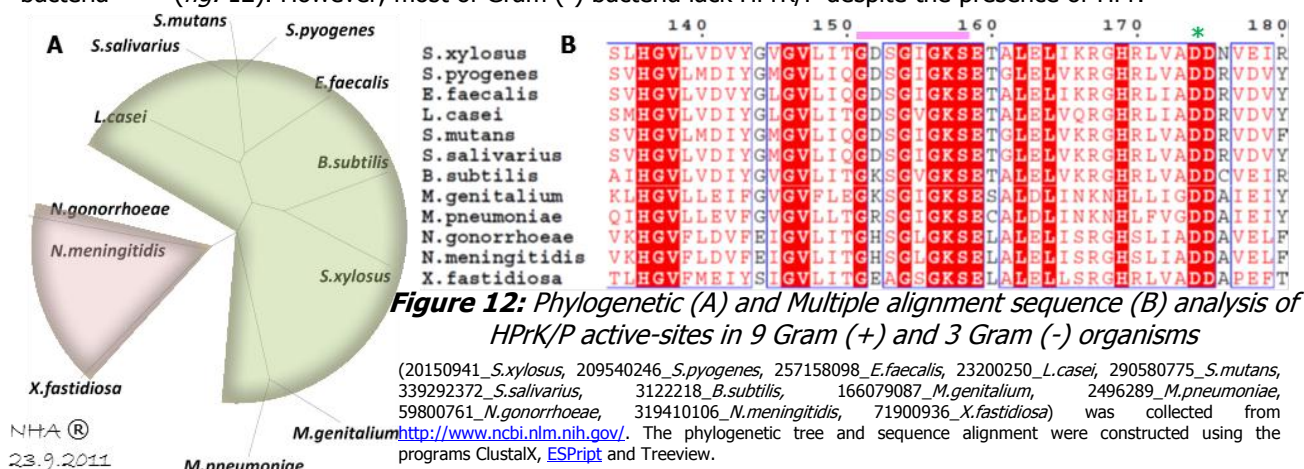
HPr (*ptsH* gene<sup>412</sup>) is phosphorylated by EI on His-15. In most low-G+C Gram (+) and a few Gram (-) bacteria, HPr can also be phosphorylated by an ATP-dependent protein kinase (fig. 12) on a seryl residue, e.g., Ser-46 in *B. subtilis*. The second regulatory phosphorylation is not part of the phosphoryl transfer to carbohydrates, but phosphorylation of the seryl residue slows the phosphoryl transfer from  $P_i$ -His-EI to HPr at least 100-fold. The fig. 11 illustrates the phosphoryl flow inside PTS, where the phosphorylation state of the PTS proteins depends on the concentration of both extracellular carbohydrates and internal PEP.



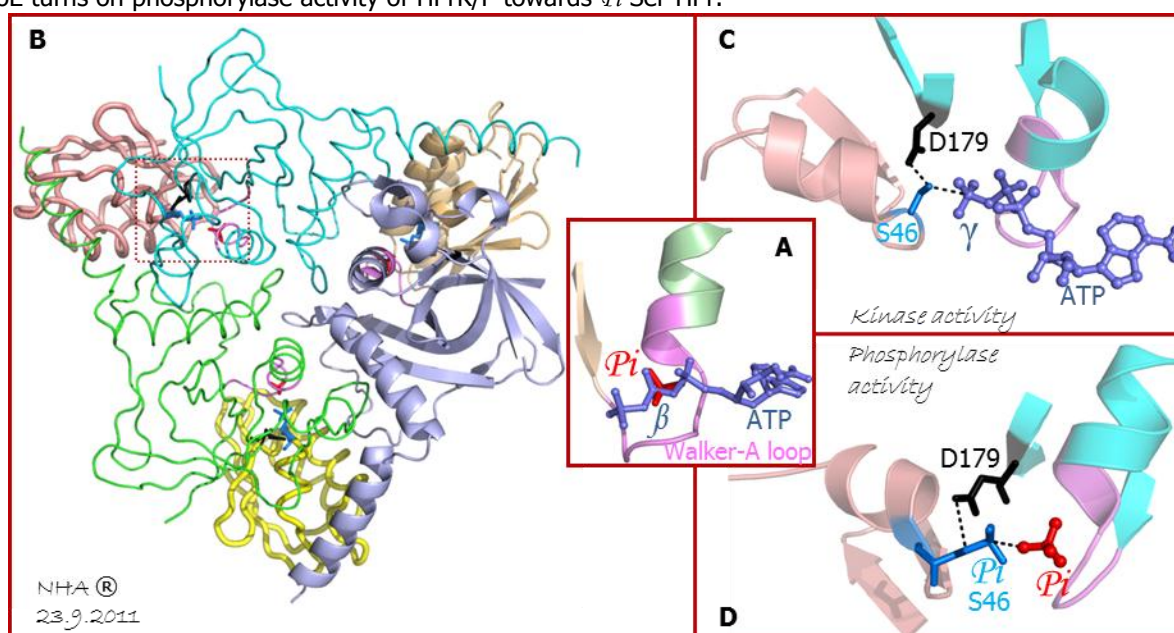
...what is HPrK/P & how does it work at molecular level?...

As mentioned above, there is a particular interest to the ATP-dependent protein kinase whose target is HPr. In 1983, Deutcher & al.<sup>17</sup> for the first time demonstrated that the phosphotransferase activity of *Streptococcus pyogenes* HPr was regulated by reversible serine phosphorylation controlled by an HPr-kinase of 20 kDa and an HPr-phosphatase of 70 kDa (*cf. tab. 1*). However, once the gene *hprK* had been cloned<sup>73,413</sup>, its protein product of 35 kDa turned out to possess bi-functional activities: HPr phosphorylation and P-Ser-HPr dephosphorylation<sup>414</sup>.

A Walker-A motif was found to be conserved in HPrK/P of different organisms, including both Gram (+) and (-) bacteria<sup>413,415</sup> (*fig. 12*). However, most of Gram (-) bacteria lack HPrK/P despite the presence of HPr.



The two antagonistic activities of HPrK/P are regulated by metabolites, such as fructose-1,6 bisphosphate (FBP) and inorganic phosphate ( $P_i$ )<sup>413</sup>. In addition, HPrK/P can use two different phosphoryl donors: ATP and pyrophosphate ( $PP_i$ ). Only the ATP-dependent kinase activity is stimulated by FBP<sup>416</sup> (*fig. 11*). The crystal structures of HPrK/P from three different organisms have been solved<sup>417</sup>, showing no similarity to other kinases families (*cf. I.1*). The  $P_i$  was bound to the Walker motif A at the site occupied by the  $\beta$ -phosphate of ATP<sup>18</sup> (*fig. 13-A* was obtained from the superposition of 2 active-sites, ATP-PEPCK-1ayl.pdb and  $P_i$ -HPrK/P-1kkl.pdb). This explains the inhibitory effect of  $P_i$  on the ATP-dependent kinase activity. However,  $P_i$  was also reported to stimulate the Hpr-phosphatase activity, which was unusual, as  $P_i$  was expected to be the product of  $P_i$ -Ser-HPr dephosphorylation. In fact,  $P_i$ -Ser-HPr dephosphorylation turned out to follow an unusual mechanism.  $P_i$  bound to the Walker motif functions as substrate and carries out a nucleophilic attack on the phosphorous atom of  $P_i$ -Ser-HPr, thus leading to the formation of  $PP_i$ <sup>418</sup> and hydroxylated HPr, which then recuperate a proton from Asp179<sup>419</sup> (*fig. 12D*).  $P_i$ -Ser-HPr dephosphorylation is therefore a phosphorolysis and not a hydrolysis reaction. In fact, it is the reversal of the  $PP_i$ -dependent HPr phosphorylation. The structures of HPrK/P in complex with HPr and  $P_i$ -Ser-HPr have also been determined and confirmed the proposed  $P_i$ -Ser-HPr dephosphorylation mechanism. The phosphatase activity previously observed in the separate protein YvoE<sup>73</sup> was then assumed to be specific to  $PP_i$  but not to  $P_i$ -Ser-HPr<sup>417</sup>. By producing free  $P_i$  – inhibitor of HPr-kinase, this pyrophosphatase YvoE turns on phosphorylase activity of HPrK/P towards  $P_i$ -Ser-HPr.

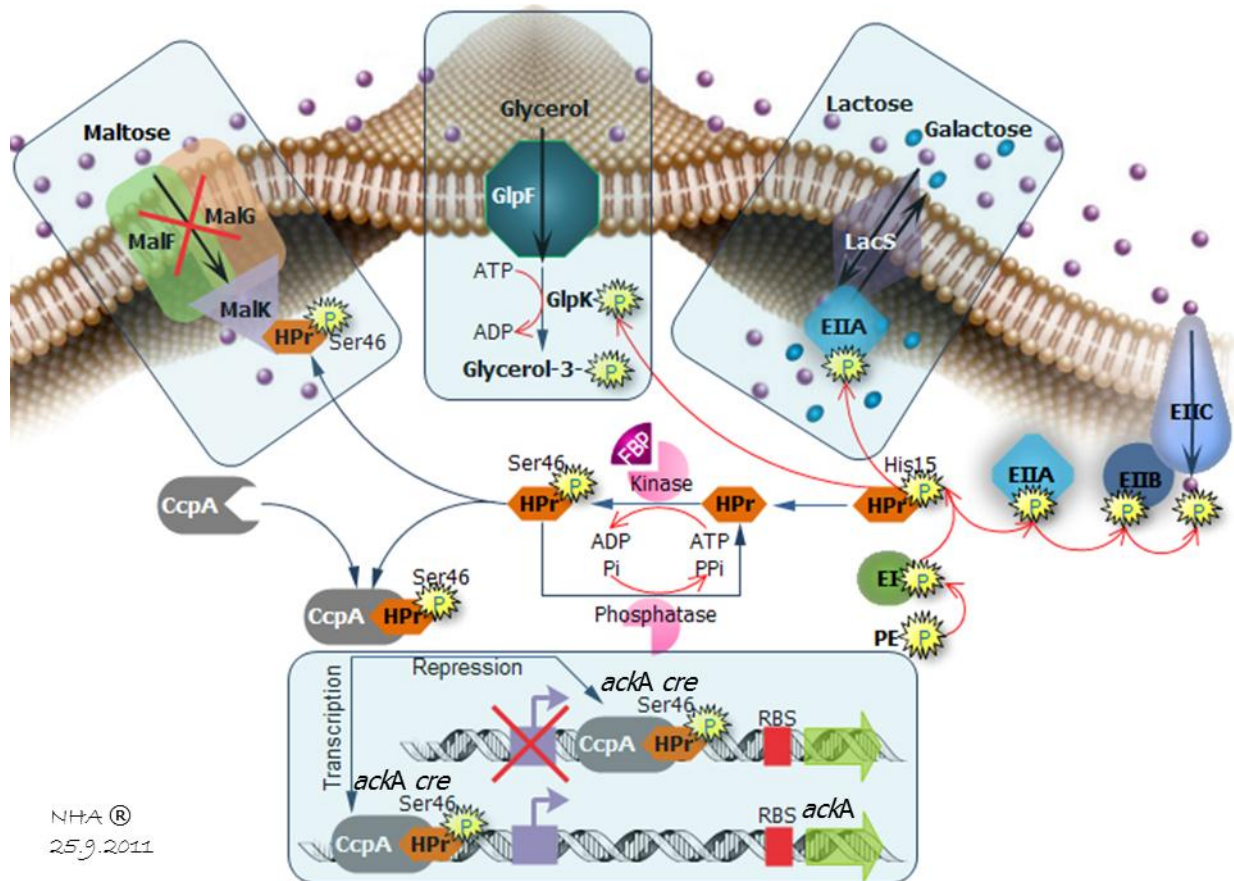


**Figure 13 : Kinase and phosphorylase catalytic mechanism of HPrK/P towards HPr**

In *fig. 13B*, HPr was presented in yellow, wheat and salmon color, the rest are 3 chains of HPrK/P. The small dashed square was zoomed in *fig. 12C* (with HPr) and *fig. 12D* (with  $P_i$ -Ser-HPr)

...what is the role of HPrK/P in PEP:PTS?...

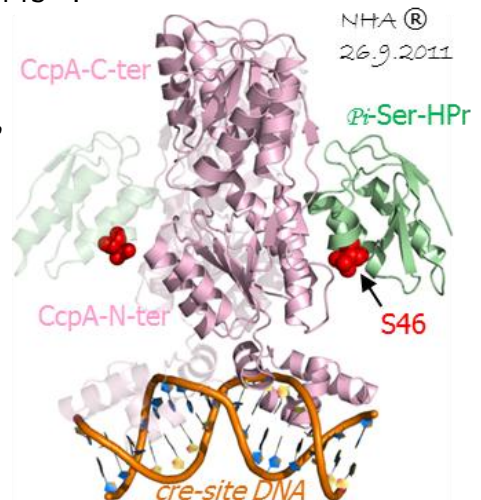
With two phosphorylatable residues, HPr exists in four different forms in HPrK/P containing organisms: HPr,  $P_i$ -His-HPr,  $P_i$ -Ser-HPr and  $P_i$ -His-Ser-HPr<sup>420</sup>. The obvious role of HPrK/P is therefore to maintain the adequate equilibrium between these forms. The high concentration of FBP present in cells growing on a rapidly metabolizable carbohydrate stimulates the kinase activity of HPrK/P and the formation of  $P_i$ -Ser-HPr. High concentration of  $P_i$  present in resting cells favors the  $P_i$ -producing dephosphorylation of  $P_i$ -Ser-HPr by HPrK/P. HPr plays a central role in carbon catabolite repression/activation (*i.e.* CCR/CCA, the phenomenon where some genes are more strongly repressed/ expressed in the presence of preferred carbohydrates) as well as in transport activity of PTS and inducer exclusion.



**Figure 14** : Role of HPrK/P in CCR/CCA, PTS transport activity and inducer exclusion

In Gram (+) bacteria, CcpA is the master regulator of carbon catabolite control, which ensures optimal energy usage under diverse conditions<sup>19</sup>. CcpA belongs to the LacI/GalR regulator family, characterized by an N-ter DNA-binding domain and a large C-ter effector-binding domain (*fig. 15*). Unlike other members of the family, CcpA needs to be activated by a corepressor protein to be able to bind to DNA. In bacilli,  $P_i$ -Ser-HPr and its homologous  $P_i$ -Ser-Crh function as such catabolite corepressor<sup>421</sup>. Crh is phosphorylated on Ser-46 by HPrK/P in the presence of FBP, but lacks His-15 and therefore cannot function in PTS<sup>422</sup>.

*B. subtilis* CcpA functions as both a transcription repressor and activator and binds to ~ 50 of the total 160 *cre*'s (catabolite response elements) to regulate ~ 400 genes (10% of the genome)<sup>20,423</sup>. CCA occurs when the *cre* is located upstream from the promoter, (e.g. *cre* for acetate kinase-encoding *ackA* gene<sup>424-426</sup>). By contrast, CCR requires a *cre* located within or downstream from the promoter (*fig. 14*). The interaction of the corepressor  $P_i$ -Ser-HPr with CcpA is highly specific, as HPr or  $P_i$ -His-Ser-HPr exhibited only very low affinity for CcpA<sup>427,428</sup>. Thus, CCA of *ackA* gene is absent from *ptsH1* mutants (lacking  $P_i$ -Ser-HPr due to Ser46Aln mutation). In agreement with this concept, *ptsH1* mutants<sup>421,429</sup> as well as  $\Delta hprK$ <sup>73,430</sup> or inactivated HPrK/P<sup>431</sup> mutants were all relieved from CCR. However, in *B. subtilis*, the releasing effect in *hprK* mutants is markedly stronger than that in *ptsH1* mutants. This difference is due to the presence of Crh which can partly substitute for  $P_i$ -Ser-HPr in CCR/CCA.



**Figure 15** : CcpA/HPr/*ackA-cre* structure complex<sup>19</sup>



$P_i$ -Ser-HPr regulates not only in CCR/CCA, but also influences the PTS transport activity, since phosphorylation at Ser-46 makes HPr a poor substrate for the His-15-phosphorylation by EI<sup>432</sup>. The hydroxyl group of Ser-46 of HPr faces the Glu-84 of EI in the protein/protein complex and electrostatic repulsion is therefore likely to be responsible for the poor affinity between enzyme I and  $P_i$ -Ser-HPr<sup>433</sup>. As a consequence of the slow phosphoryl transfer between EI and  $P_i$ -Ser-HPr, the V267FP\_i-Ser-HPr failed to transport PTS sugars<sup>434</sup>.

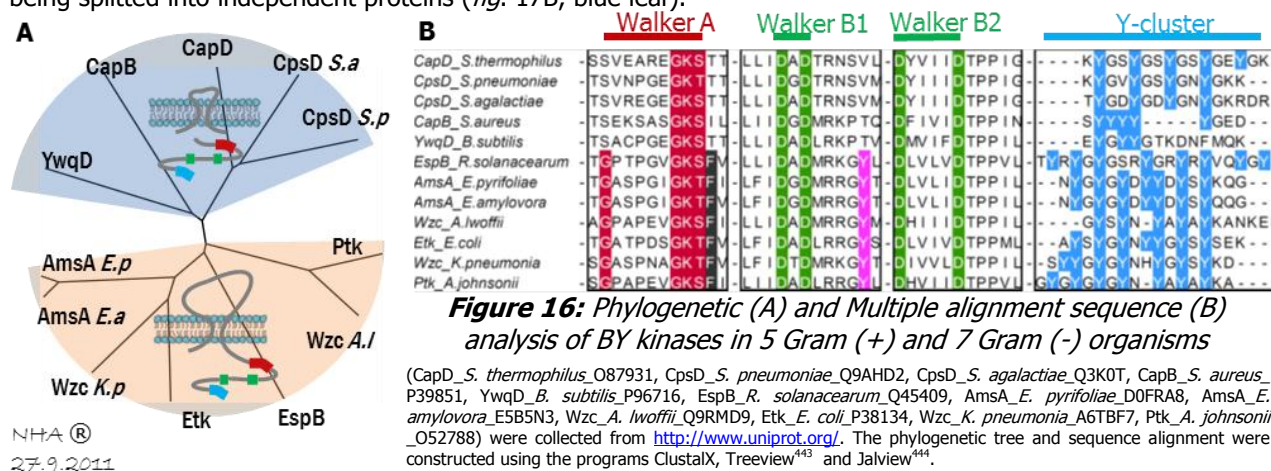
$P_i$ -Ser-HPr also probably interacts with certain non-PTS carbohydrate transport systems, such as the maltose transporter MalF-MalG from *L. casei*, and thereby inhibits their transport activity (i.e. inducer exclusion phenomenon) (fig. 14, top left). By contrast, maltose uptake by mutants lacking  $P_i$ -Ser-HPr (*ptsH1*<sup>435</sup> or  $\Delta$ *hpK* or inactivated *hpK*<sup>436</sup>) was not affected by the presence of glucose. In other words, lag phase during diauxic growth in a mixture of glucose and the non-PTS sugar maltose disappeared in these mutants. Phosphorylation of LacS by either  $P_i$ -Ser-HPr and doubly  $P_i$ -Ser-His-HPr stimulates the lactose/galactose exchange reaction in *S. thermophilus*<sup>437</sup> (fig. 14, top right). In the presence of rapidly metabolizable PTS sugars, the low level of  $P_i$ -His-HPr does not allow sufficient phosphorylation of glycerol kinase GlpK, leading to the inactivation of GlpK and to inducer exclusion<sup>438</sup> (fig. 14, top center).

### I.2.3. Bacterial Tyrosine Kinases (BYks)

The second class of Walker motif-containing bacterial protein kinases is Tyr kinase family (i.e. BYk). Due to the very low occupancy of tyrosine phosphorylation sites in nature<sup>40,439</sup> which challenged available detection techniques and analytic tools, it was not until the late 1990's that the first BYk, Ptk from *A. johnsonii* was discovered<sup>24,72</sup>. Since then, homologs of Ptk have been detected in a large number of Gram (-)<sup>440,441</sup> & Gram (+) bacteria<sup>74,442</sup>, and is seemingly ubiquitous in bacteria<sup>319</sup> but absent in *Eukarya*<sup>26,108</sup>. So far, of ~ 20 characterized bacterial tyrosine kinases<sup>108</sup>, only three are not homologs of Ptk (cf. I.1.3.3 & I.1.3.4 for DivL, MasK WaaP). Despite their omnipresence, BYks are limited to only a few copies (usually one or two) per bacterial genome<sup>96</sup>.

...what is BYks?...

A typical *Proteobacteria* BYk consists of an N-ter transmembrane domain followed by a cytosolic catalytic domain containing the ATP-binding site, and a C-ter tail containing autophosphorylatable tyrosine cluster<sup>108</sup> (fig. 17A, light-orange leaf). In *Firmicutes*, the BYk gene has been split in two, encoding separately the transmembrane domain and the cytosolic kinase, both of which maintain a tight functional interaction despite being splitted into independent proteins (fig. 17B, blue leaf).



...in which cellular pathways are BYks implicated?...

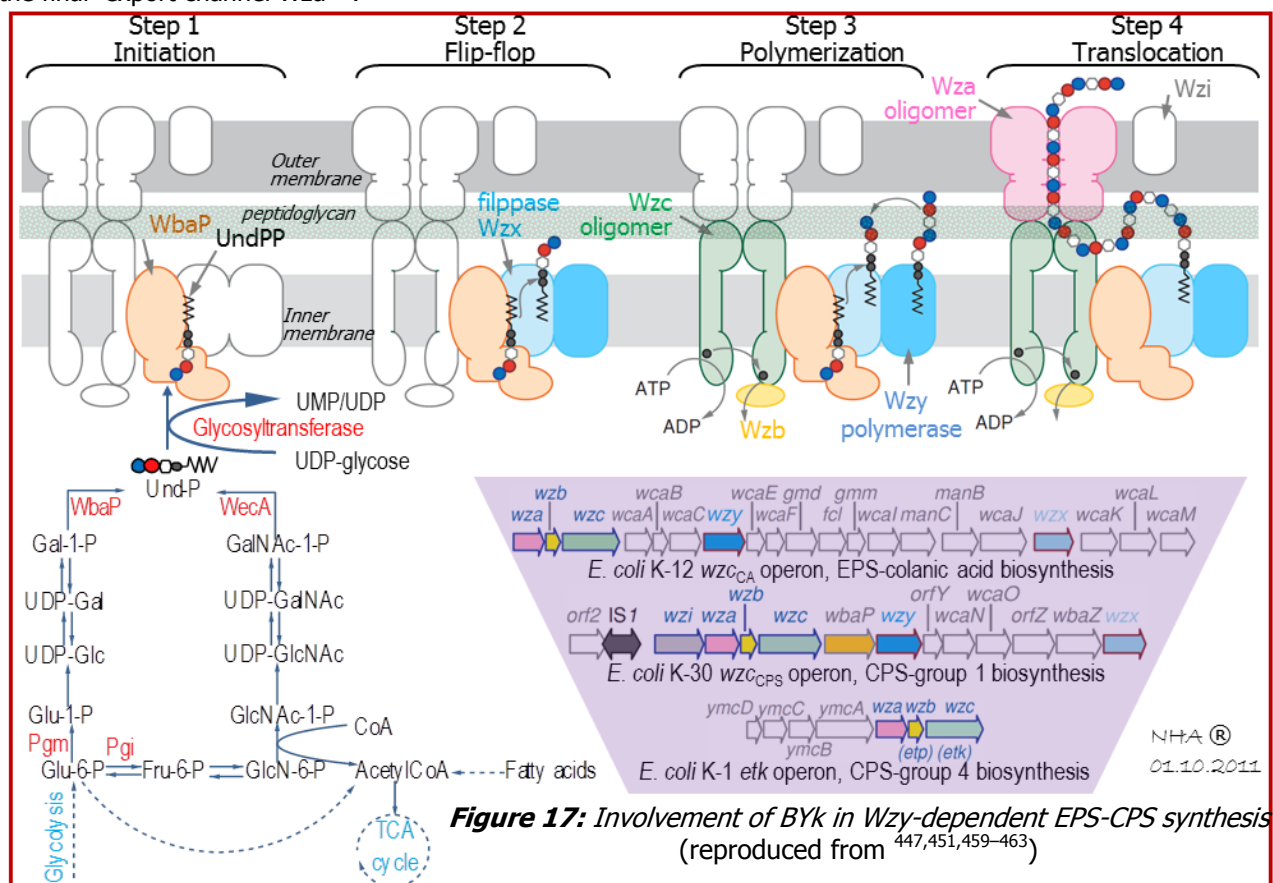
The first phase of BYk research (up to 2003) marked the involvement of BYk's auto-kinase activity in synthesis and export<sup>109,318,327,359,402,404,445,446</sup> of extracellular and capsular polysaccharides (i.e. EPS and CPS). These polysaccharides play critical roles in interactions between bacteria and their environment, and in many pathogens, they promote virulence across the outer membrane<sup>447</sup>. In 1999, Whitfield & al.<sup>448</sup> proposed an updated classification of *E. coli* CPS comprising 4 groups based on genetic and biosynthetic criteria. In this bacteria, Wzc<sub>CPS</sub><sup>449</sup> (strain K30), Etk<sup>74</sup> (strain K12 and K30) and Wzc<sub>CA</sub><sup>404</sup> (strain K12) are 3 BYks implicating in synthesis of CPS-group 1, group 4 and EPS-colonic acid respectively, via Wzy-dependent pathway<sup>448</sup> (fig. 17):

CPS-group 1, 4 and EPS-colonic acid are synthesized from diphospho-undecaprenol precursor<sup>450</sup> (i.e. Und-PP) which is the final product of a nucleotide-sugar metabolism pathway. Before the addition of glycose to the membrane-associated lipid intermediate undecaprenyl phosphates (i.e. Und-P)<sup>451</sup>, WbaP or WecA membrane-enzymes are required for transferring Galactose-1-P or Galactose-N-acetylhexosamine-1-phosphate to Und-P (fig. 17).

Und-PP units are assembled at the interface between the cytoplasm and the inner membrane (step 1). Newly synthesized Und-PPs are then flipped across the inner membrane in a process requiring Wzx (step 2). This provides the substrates for Wzy-dependent polymerization wherein the polymer grows by transfer of the growing chain to the incoming Und-PP unit (step 3). Polymer is translocated by Wza, which likely acts as a channel. Wzi is unique to CPS-group 1 and appears to be involved in modulating surface association (step 4).

Involvement of BYKs in Wzy-dependent EPS-CPS biosynthesis takes place in the third step by controlling high-level polymerization of CPS/EPS, and is seemingly strain-specific. Indeed, tyrosine auto-phosphorylation of Wzc<sub>CPS</sub> was shown to positively regulate the CPS-group 1 polymerization in *E. coli* K-30<sup>440</sup>, whereas in *E. coli* K-12, the colanic acid synthesis is decreased when Wzc<sub>CA</sub> is phosphorylated<sup>109</sup>. In agreement with this idea of strain-specificity, tyrosine auto-phosphorylated CpsD reduces CPS polymerization in *S. pneumoniae* RX1 strain<sup>452</sup> but positively affect CPS production in *S. pneumoniae* D39 strain<sup>453</sup>. Since mutations of *wzc* and its cognate phosphatase *wzb* both result in an acapsular phenotype, it was suggested that Wzc function requires its cycling between phosphorylated and nonphosphorylated forms<sup>440</sup>.

The precise function of BYKs is still unknown. One possibility is that these proteins really are "co-polymerases" and interact with Wzy. However, *wzc* mutants also result in dramatic reduction in Gal-1-P transferase activity of WbaP<sup>447</sup>, suggesting a direct Wzc-WbaP interaction or a structural role of Wzc in the Wzy-dependent complex machinery including WbaP. In agreement with this later proposal, interactions between Wzc and the integral membrane flippase Wzx<sup>454</sup> as well as the outer membrane exporter Wza<sup>455</sup> were also reported. Crystal octameric ring-shaped structures of both Wza<sup>456</sup> and Wzc cytoplasmic<sup>323</sup> reinforce the hypothesis of a continuous large hollow pore spanning both inner and outer membrane which controls polymerization and export of CPS/EPS through cyclic phosphorylation/ dephosphorylation. Wzc might therefore, together with Wzx flippase, participate in transporting incoming precursor across the inner membrane<sup>323</sup>. In congruence with this hypothesis, it was reported that Wzc regulates not only the length but also the amount of CPS<sup>457</sup> thus suggesting its regulatory role in the quantity of exported precursors. Alternatively, it has been proposed that Wzc serves as a phosphorylation-dependent gate, which allows only precursors of enough length to enter into the final export channel Wza<sup>458</sup>.



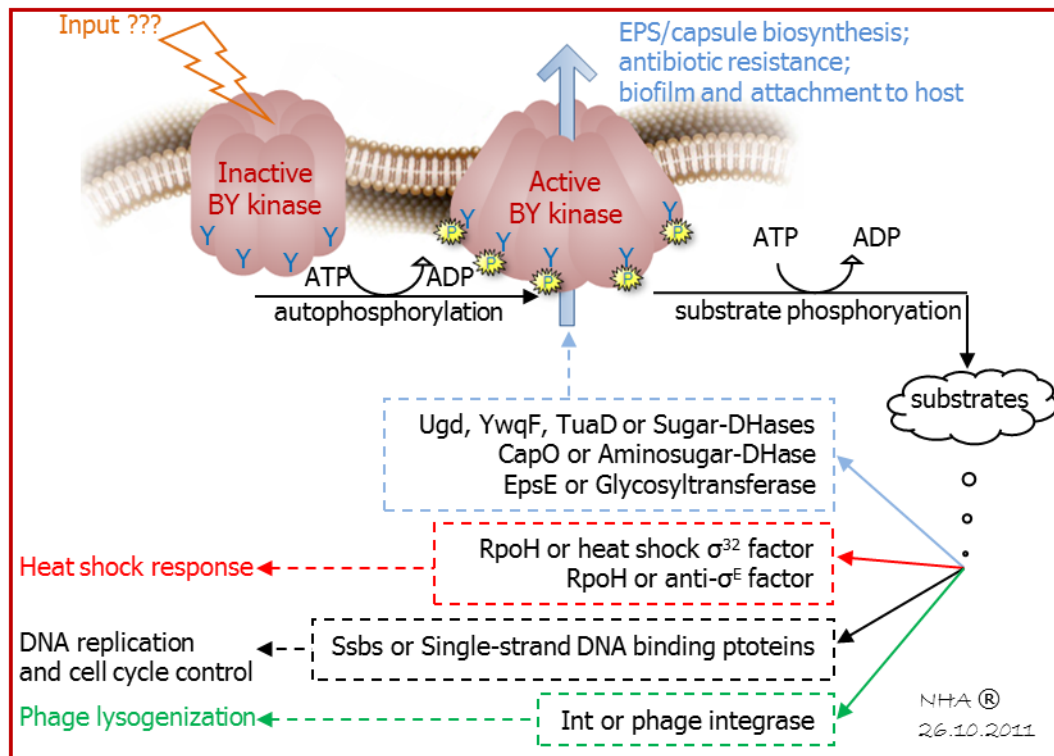
From 2003, the field renewed its blood when other endogenous protein substrates were found to be phosphorylated on Tyr-residues by BYKs. Nowadays it turns out that Tyr-phosphorylation in bacteria regulates not only EPS-CPS production but also many other cellular processes. The following *fig.18* summarises already known functions of BYKs.

RpoH and RseA are *E. coli* heat shock  $\sigma$  and anti- $\sigma$  factors that were found to be Tyr-phosphorylated by the BYK Etk, suggesting the role of Etk in heat shock response<sup>325</sup>.

Wzc in *E. coli* was thought to down-regulate the activity of HK022 and  $\lambda$  intergrase proteins (Int) via transphosphorylation. Overexpression of Wzc resulted in a significantly reduced lysogenization. However, Int phosphorylation *in vivo* does not require the presence of Wzc, suggesting that another tyrosine kinase is capable of phosphorylating these intergrases<sup>464</sup>.

In *S. thermophilus*, the glycosyltransferase EpsE is activated via Tyr-phosphorylation by its cognate BYK EpsD. In *S. aureus*, UDP-*N*-acetylmannosamine deshydrogenase activity of the capsular precursor CapO is activated via Tyr-phosphorylation by the BYK-B2. The same phenomenon was observed with the Ugd family in both *E. coli* and *B. subtilis*: Ugd<sub>EC</sub> was Tyr-phosphorylated by Wzc<sub>CA</sub><sup>319</sup>; two Ugd<sub>BS</sub> (YwqF and TuaD) were phosphorylated by PtkA in the presence of membrane protein TkmA (YwqC)<sup>311</sup>. In both cases, UDP-glucose

deshydrogenase activity of these substrates was increased after phosphorylation, resulting in the formation of UDP-glucuronic acid. The latter is the teichuronic acid precursor which participates not only in colonic acid and group 4 capsule biosynthesis, but also in the formation of L-Ara-4N (*i.e.* 4-amino-4-deoxy-L-arabinose) involved in polymyxin resistance phenomenon<sup>321</sup>.



**Figure 18:** Involvement of BYK in different cellular pathways

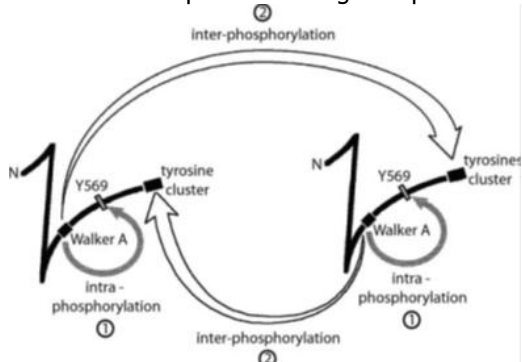
PtkA in *B. subtilis* is also capable of phosphorylating two single-strand DNA binding proteins, SsbA and SsbB (YwpH) in the presence of the membrane activator TkmA<sup>312</sup>. Defects in initiation of DNA replication and chromosome distribution in depleted *ptkA* gene strain of *B. subtilis*<sup>405</sup> suggest the involvement of this BYK in DNA metabolism.

Interestingly, BYK is not only capable of changing its substrate activity, but also ensures the correct subcellular localization of its targets<sup>406</sup>. Phosphoproteomic data of *B. subtilis*<sup>28</sup> revealed nine new Tyr-phosphorylated proteins that were confirmed to be *in vitro* substrates of PtkA. Some of them e.g. Ldh, YnfE and YvyG are found to co-localize with PtkA, whereas the localization of several others, e.g. enolase, YjoA, Ugd and SsbA, was dependent on their phosphorylation status and not the direct interaction with the kinase itself.

Briefly, the emergent phosphorylation networks conducted by BYKs and their substrates promise significant complexity and there are still much open land research in this field, both in term of intracellular signaling pathway exploitation as well as elucidation of signal input triggering autophosphorylation.

...how does BYK works at the molecular level ?...

Since different domain organization was found in *Proteobacteria* and *Firmicutes* BYKs, it was not surprising that Gram (-) and Gram (+) bacteria used remarkably different mechanisms of Tyr-phosphorylation. Given that the *Firmicute* BYK was composed of two distinct domains, a transmembrane domain containing an extracellular loop, and a cytosolic kinase domain possessing Y-cluster and Walker A, B1, B2 motifs, it was proposed that the membrane domain plays a role of activator for the cytosolic kinase domain, and the extracellular loop serves as signal input sensor. Whereas the latter hypothesis is still obscure<sup>26</sup>, lots of data have



**Figure 19:** Autophosphorylation mechanism of Wzc<sub>CA</sub> in *E. coli* K12 via a two-step process<sup>318</sup>

actually proven the activation of cytoplasmic kinase via interacting with their membrane partner<sup>311,359,465</sup>. More precisely, interaction between inactive kinase and the cytoplasmic C-ter peptide of the membrane domain is sufficient for triggering kinase activity<sup>311,465</sup>. In the same way, the cytoplasmic domain of almost all *Proteobacterial* BYKs is capable of developing kinase activity independently from the periplasmic N-ter domain<sup>318</sup>.

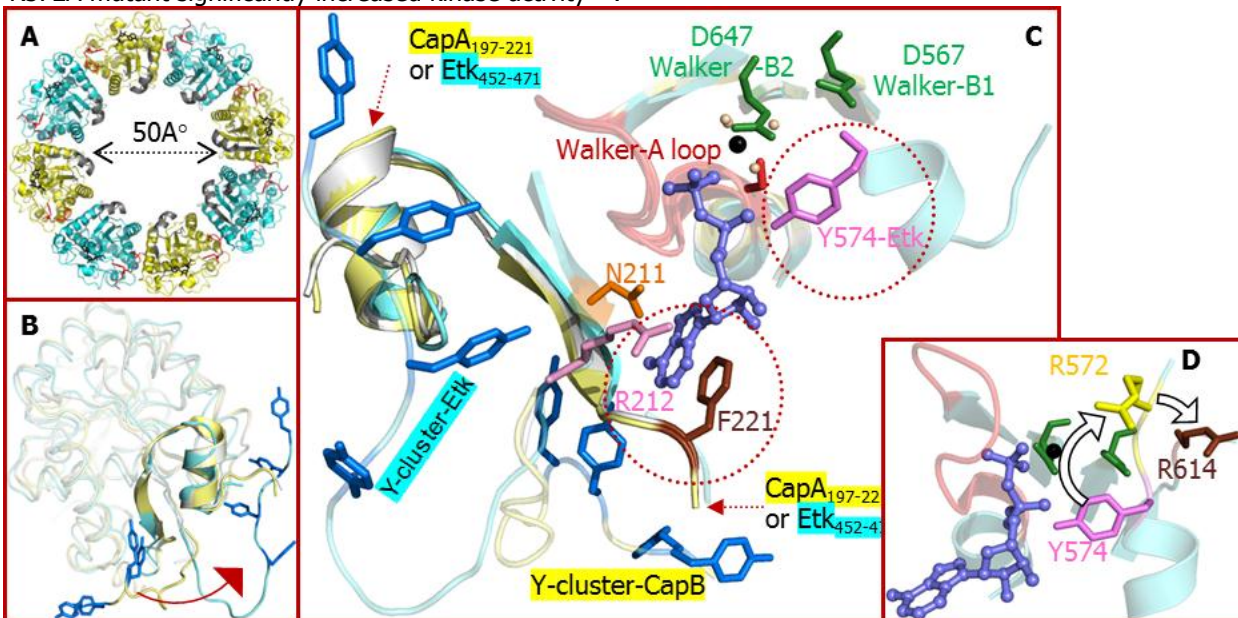
One of the characteristics of BYKs is their capacity of autophosphorylating a number of Tyr residues within Y-cluster. In *Firmicutes*, this autophosphorylation undergoes a single-step mechanism of either inter-or intra-phosphorylation. For instance, *S. pneumoniae* Cps2D purified in *E. coli* is already present in



autophosphorylated form which is capable of dephosphorylated Cps2D in the absence of ATP<sup>442</sup>.  
phosphorylating onto another

Whereas in *B. subtilis*, even in the presence of ATP, phosphorylated YwqD failed to transphosphorylate other inactive YwqD molecules lacking Asp residues of Walker B1 motif<sup>311</sup>. In *Proteobacteria*, a number of BYks possess an internal Tyr which differs from Y-cluster and locates just after the Walker B1. At the beginning, following the results found for Wzc<sub>CA</sub> in *E. coli* K12, it was proposed that the first step of intra-phosphorylation (where ATP bound to Walker A motif transfers the phosphate onto the internal Tyr) was necessary to activate the second step of inter-phosphorylation (where the phosphate from Walker A binding ATP was transferred onto Y-cluster of another kinase molecule) (fig. 19)<sup>318</sup>. However, in other cases, this internal Tyr is either not phosphorylated or does not influence the Y-cluster phosphorylation<sup>387, 413</sup>, including the Wzc<sub>CPS</sub> in *E. coli* K30<sup>449</sup> or is even absent in other cases, including the *S. aureus* CapB (see fig. 16). If there is not an artifact, Tyr-phosphorylation therefore would seemingly have been a dynamic event strain specific and by now it is still hard to generalize the autophosphorylation mechanism to BYks.

Interestingly, in 2008, the structure of the cytoplasmic domain of *E. coli* K12 Etk<sup>467</sup> revealed the role of internal Tyr at molecular level. The side chain of this Y574 of non-phosphorylated form directly points to the active site of Etk, thus prevents the binding of Mg<sup>2+</sup>. Upon phosphorylation of Y574, its negatively-charge side chain undergoes a rotation provoked by electrostatic attraction towards the well-conserved positively-charged R614 (fig. 20C, 20D), hence unblocks the catalytic site. Unfortunately, the lack of phosphorylated Etk structure in which *Pi*-Y574 would be in the open or active conformation did not help to wrap the Tyr story. In 2009, molecular dynamics simulation confirmed the molecular switch involving *Pi*-Y574 and R614 and furthermore brought a previously unidentified residue, R572 into sight. This residue was proposed to be a modular key slowing down the opening of the active site (fig. 20D). Biochemical data supported this hypothesis since Etk-R572A mutant significantly increased kinase activity<sup>468</sup>.



**Figure 20:** Overview on autophosphorylation mechanism in BYks

- A: *S. aureus* CapAB unphosphorylated octamer with membrane activator CapA in grey and kinase CapB in cyan and yellow. Y-cluster of each subunit (red) points to the nucleotide (black) in the active site of the neighbouring subunit. This picture is copied from<sup>86</sup>.  
 B: Spatial occupation of Y-cluster is seemingly strain specificity. Phosphorylated CapB in fuzzy grey (3BFV.pdb), unphosphorylated CapB-K55M in fuzzy yellow (2VED.pdb) and unphosphorylated Etk in fuzzy cyan (3CIO.pdb) are superimposed. While the kinase cores (fuzzy) and membrane activators (net) of CapAB and Etk are almost superposable, the two Y-clusters are distinguishable.  
 C: Activation of CapA (net) towards kinase core (fuzzy) reveals interactions between F221 of CapA and the base moiety of ADP. The latter is also stabilized by N211 and R212 of CapB kinase core. In Etk, the internal unphosphorylated Y574 acts as a close gate blocking access of Walker B1-D567 (green) and perhaps D569 (not illustrated) into the active site.  
 D: Swing of Y574 towards R614 upon phosphorylation is controlled by the intermediate residue R572 in Etk.

Recent structural studies brought important insights into oligomeric structures of transmembrane BYks. Autophosphorylation is proposed to be the mechanism for oligomer dissociation. Before phosphorylation event, the Y-cluster of each subunit binds into the active site of the neighbouring molecule (fig. 20A). It was supposed that once trans-phosphorylated by the adjacent subunit, this flexible Y-cluster left the active site, hence resulting in octamer dissociation<sup>86</sup>.





*Results  
&  
Discussion*

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## II. Results-Discussion

### II.1. Evidence for a new class of protein kinases in bacteria

#### II.1.1. Evidence for a new class of enzymes present exclusively in bacteria



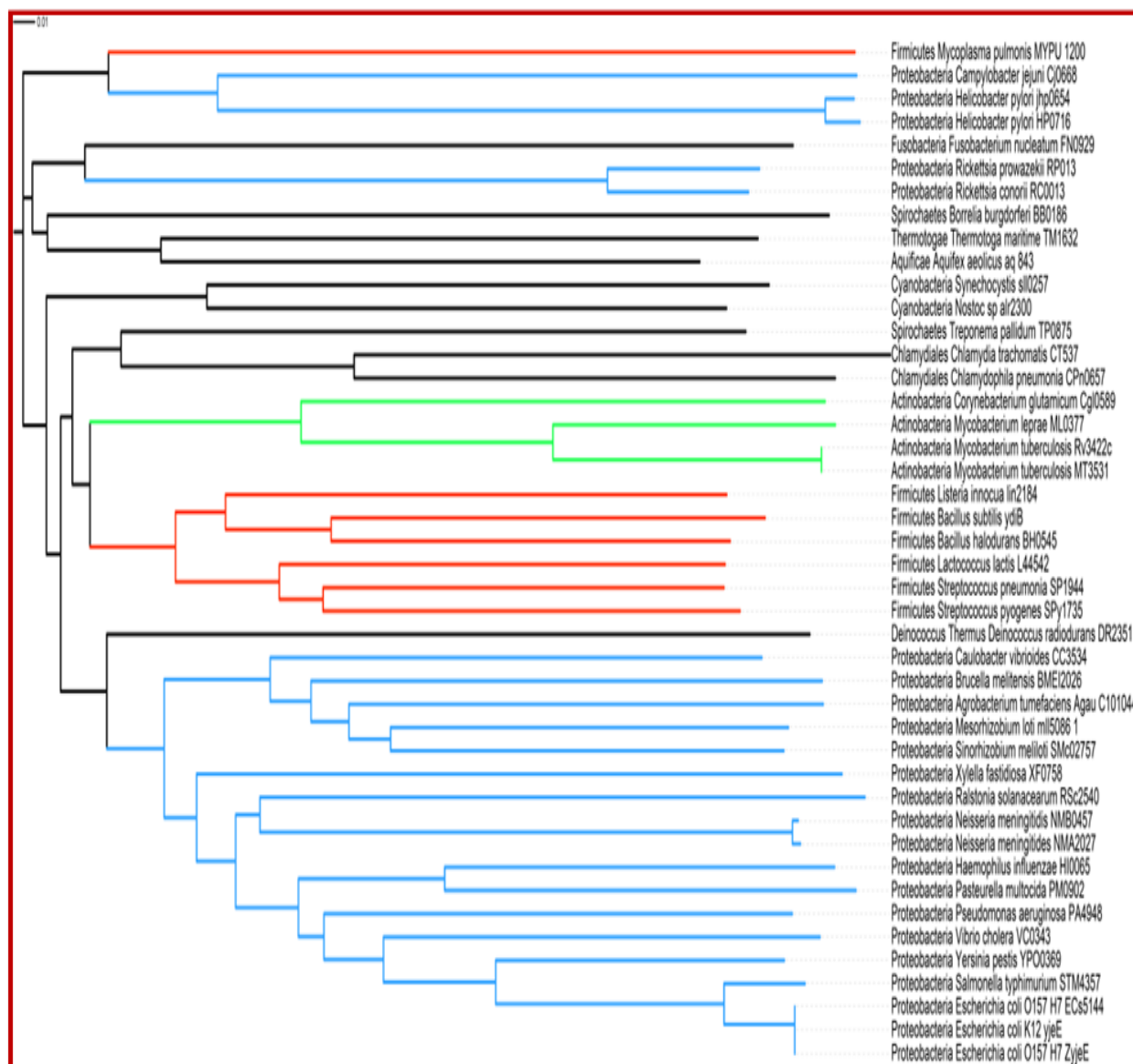
*Functional genomics is the new rock and roll*<sup>469</sup>!

*The great challenge in biological research today is how to turn data into knowledge. I have met people who think data is knowledge but these people are then striving for a means of turning knowledge into understanding*<sup>2</sup>.



If the year of 1995 marked the first sequenced genome of a free-living organism, *Haemophilus influenzae*, at the end of 2011, full genome sequencing becomes normal, even 'blasé' science with the expected efficiency of 1000 genomes per month<sup>1</sup>. The major task that the scientific community is now facing is to turn data into knowledge<sup>2</sup>. In the first step, computational methods including sequence comparison, phylogenetic patterns and gene neighborhoods are inevitable to establish homolog network of each putative proteins in order to retrace its possible cellular function. However on average, there is no clear functional prediction for at least one-third of genes in most genomes<sup>3</sup>. These genes are either homolog-absent (true orphans) or homologous to uncharacterized gene products from other genomes (orphans).

One of the most intriguing uncharacterized protein families regroups those found in many distantly related organisms. One such case includes a conserved family of small proteins, homologous to YjeE in *E. coli*, called UPF0079 in UniProtKB<sup>4</sup> or COG0802 in the COG database<sup>5</sup>. In the *fig. 21A*, we presented 44 members of this family which spread out 10 phyla of bacterial kingdom.



**Figure 21-A:** Widespread distribution and conservation of COG0802 family in the Kingdom of bacteria

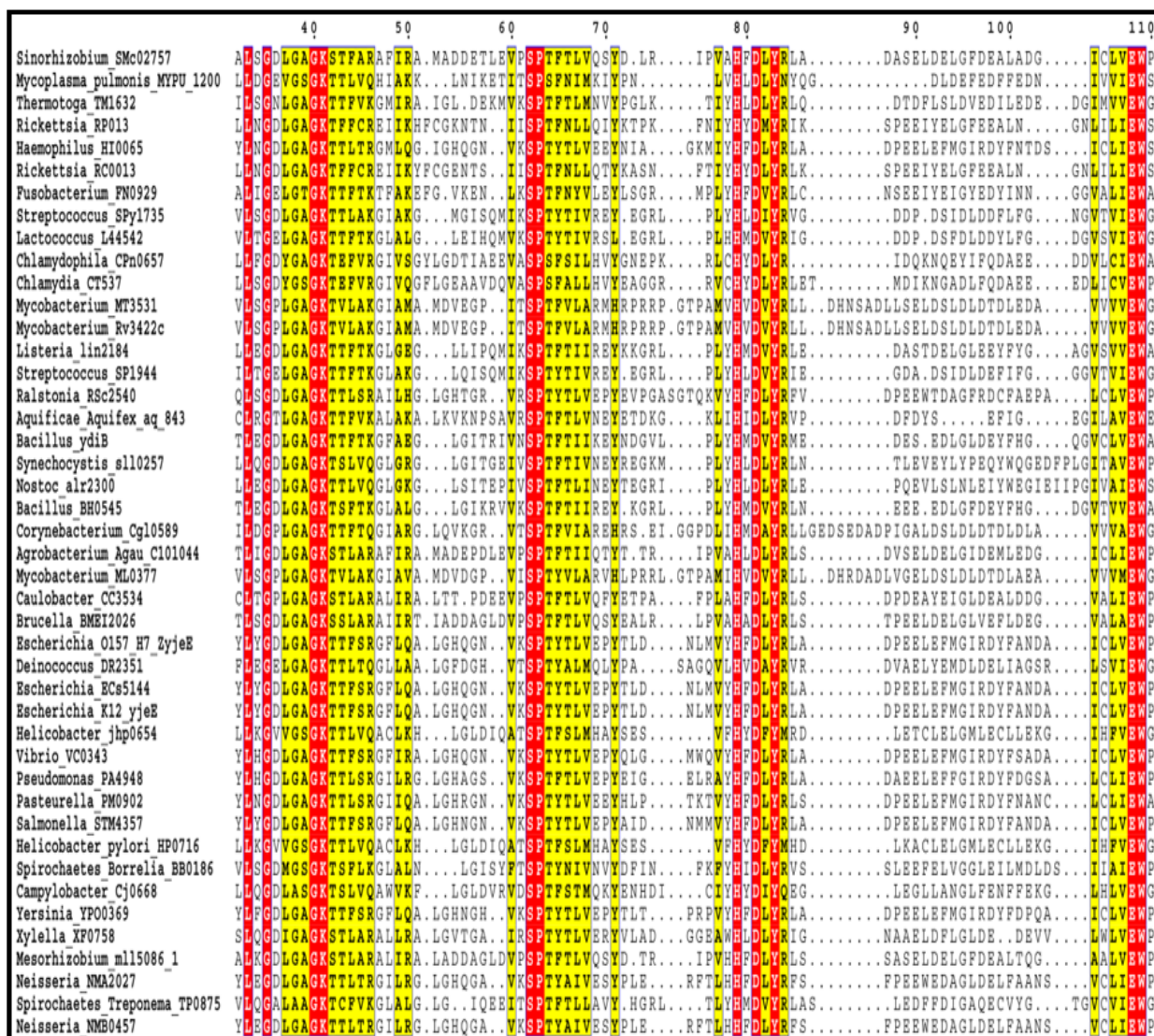
44 COG0802 proteins were collected from COG database.

Phylogenetic tree was established via Treeview<sup>470</sup> and iTOL<sup>471,472</sup> programs

This family was mentioned for the first time by Galperin in 2001<sup>6</sup> in purpose of illustrating the power and limitations of comparative genomics in deducing functions of uncharacterized proteins. Based on the conserved Walker A motif (Gx<sub>4</sub>GKT/S, *fig. 21B*) which is a fingerprint to detect nucleotide-binding proteins, this protein family has been annotated as 'probable ATP-binding protein' in UniProtKB, and 'predicted ATPase or kinase' in COG database.

At the beginning, it was thought that this new uncharacterized protein family was absent in *Mycoplasma* and *Ureaplasma*, suggesting its role in cell-wall turnover<sup>6</sup>. However, with the new coming sequenced genomes, at the writing time of this manuscript, at least 30 of the total 3237 UFP0079-homologs are of *Mycoplasma* origine. It is noteworthy that still none of these total putative homologs comes from eukaryotes, rendering this family a promising target in antimicrobial drug development<sup>7</sup>.

The widespread distribution and conservation of this family of unknown function is implicit evidence for our lack of understanding of some basic cellular process. Bioinformatics can give some predicted clues for the general biochemical properties of these proteins. However, most of the time this approach is not sufficient and follow-up biochemical characterization is required to pinpoint the physiological function(s) of these new conserved proteins.



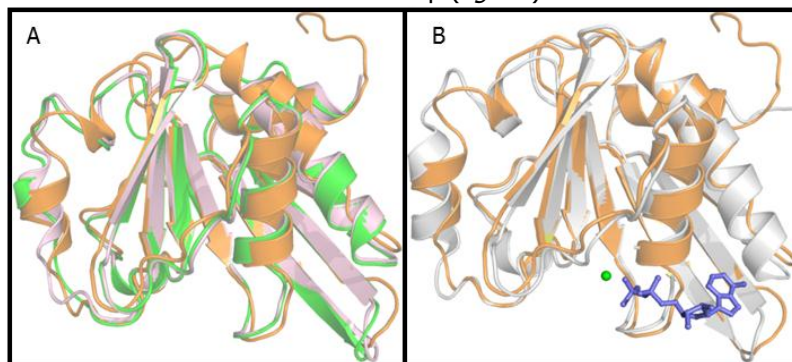
**Figure 21-B:** Potential sequence alignment of COG0802 family in the Kingdom of bacteria

44 COG0802 proteins were collected from COG database.

Multiple sequence alignment was produced by ClustalX<sup>424</sup> and ESPrnt<sup>425</sup>

## II.1.2. Structural analysis reveals new fold among ATP-binding proteins

In 2002, Teplyakov & al.<sup>8</sup> solved the crystal structure of YjeE in *Haemophilus influenzae*, the first and still the only structure of UPF0079 family till now. On waiting for the availability of other structures, we processed the modelization of *B. subtilis* YdiB based on the disponible structure of YjeE. Given that the sequences of these two proteins share 31% of identity, it was not surprising that the obtained model YdiB fit well the template YjeE, especially in the core level around the Walker A loop (fig. 22).



**Figure 22:** Modelization of *Bacillus subtilis* YdiB based on *Haemophilus influenzae* YjeE

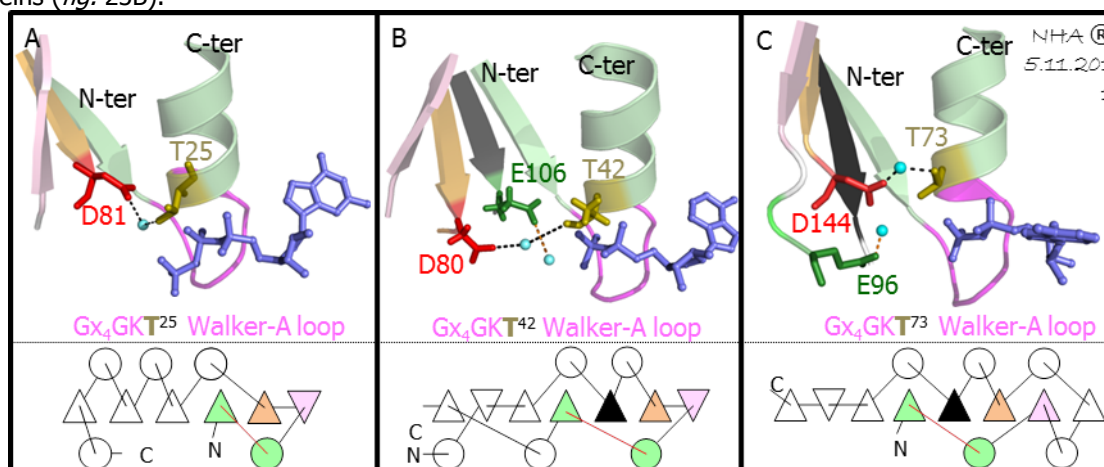
Based on the chain C of YjeE (1htw.pdb)<sup>8</sup>, A: the model apo-structure of YdiB *B. subtilis* was constructed by 3 different methods, Modeller<sup>473</sup>, ESyPred3D<sup>474</sup>, Swiss3D<sup>475</sup>; B: the model of nucleotide-binding YdiB was constructed by superposing ADP-bound YjeE (white) and Modeller apo-structure of YdiB using Pymol 1.3 software.

The evidence of a new class of enzyme one more time was confirmed when we analysed the topology of YdiB model in comparison with other nucleotide-binding proteins or more precisely Walker A-containing proteins. In I.2.1 of *Literature review*, we have already described two typical folds of Walker A proteins.

The first one, represented by EF-Tu GTPase, where the  $\beta$  strand at N-ter of Walker A motif was followed by two other adjacent antiparallel  $\beta$  strands (fig. 23A, green, orange and pink strands respectively). In this fold, the  $\beta$  strand of Walker B motif (orange) is next to the  $\beta$  strand that precedes the Walker A motif (green). This fold is referred to KG (i.e. Kinase-GTPase group) according to Leipe & al.'s classification<sup>410</sup> of P-loop NTPases.

The second typical fold is presented by RecA ATPase, with one additional  $\beta$  strand inserted between the motif A-binding  $\beta$  strand and the motif B-containing  $\beta$  strand (fig. 23C). The latter is followed by another parallel  $\beta$  sheet to form the scaffold of 4 parallel  $\beta$  strands. This fold is referred to ASCE (for Additional Strand, Catalytic E) according to Leipe & al.'s classification<sup>410</sup> of P-loop NTPases.

YdiB model shares features with both of these two folds. It contains the 4  $\beta$  sheet scaffold like RecA but the final strand is antiparallel like in the case of EF-Tu. YdiB contains also a Glutamate residue (E106) susceptible to activate a water molecule for nucleophile attack to the  $\gamma$  phosphate of nucleotide, similar to E96 in case of RecA. It is noteworthy that the KG group show numerous deviations from the hhhhD signature of the classical Walker B motif, including frequent D to E substitutions<sup>410</sup>. In YdiB, both "hypothetical Walker B strand" (orange) and "hypothetical additional strand" (black) contain a series of hydrophobic residues to form the hhhhD80 and hhhhE106 signature respectively. The assignment of Walker B strand in YdiB is therefore still a subject of controversy. Moreover, the first N-ter  $\beta$  strand of YdiB is next to its C-ter  $\alpha$  helix, thus bringing N-ter adjacent to C-ter, another unique property of YdiB in regard to other Walker A loop-containing proteins (fig. 23B).



**Figure 23:** New fold of YdiB family among Walker A-containing family

Structural topology of A: KG group, represented by EF-Tu (1exm.pdb); B: YdiB model; C: ASCE group, represented by RecA (1u94.pdb). In each case,  $\beta$  strands are presented as triangle and  $\alpha$  helix are presented as circles. The Walker A loop (magentas) connects a  $\beta$  strand (pale green) and an  $\alpha$  helix (pale green). The Walker B strand is in bright orange; the additional strand is in grey-black and the strand next to Walker B strand is in pink. Water molecules are in cyan. ADP and conserved residues (Threonine, Aspartate, Glutamate) are presented as stick.



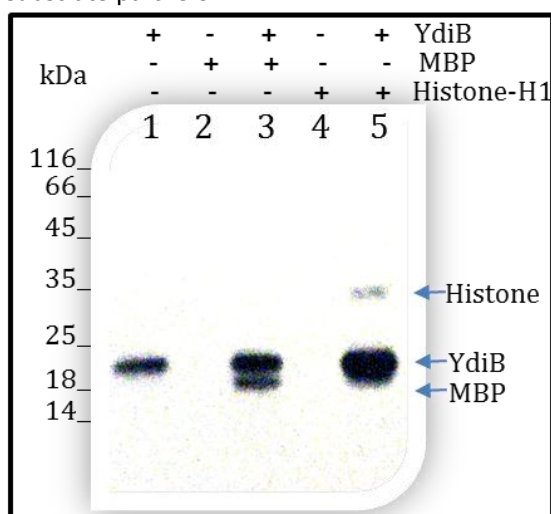
This structural analysis of YdiB and YjeE<sup>8</sup> consolidates the evidence of a new class of enzyme which possesses characteristics of both two major known folds of nucleotide-binding proteins. Together with the fact that this family UF0079 is extremely well-conserved among bacterial kingdom, one can expect that it constitutes one of the most ancient nucleotide-binding protein families that existed before the divergence into different branches of nucleotide-binding proteins as GTPases, Kinases, or ATPases.

### II.1.3. Auto-kinase and Phosphotransferase activity of YdiB

Since 2002, several groups have been reporting biochemical data on UFP0079 proteins from *H. influenzae*, *E. coli* and *B. subtilis*. In term of activity, they found agreement on a very weak ATPase activity of these proteins. The  $k_{cat}$  of *E. coli* YjeE<sup>9</sup> and *B. subtilis* YdiB<sup>10</sup> were found in the same range of 12 h<sup>-1</sup> and 10 h<sup>-1</sup> respectively. Whereas that of *H. influenzae* YjeE had been first reported to be 25 h<sup>-1</sup> by Teplyakov & al.<sup>8</sup> and then was revised by Allali-Hassani in Brown's group<sup>9</sup> to be much more weaker, of 1.2 h<sup>-1</sup> of magnitude.

It is worth noting that, stable dimeric form of YdiB was observed in *B. subtilis* extract and oligomeric forms of *E. coli* YjeE were detected by *in vivo* formaldehyde cross-linking. Additionally, oligomerization seemingly disfavors ATPase activity of YdiB which is inherently weak<sup>10</sup>. Given the previous suggestion that "YjeE-like" family might constitute one of the most ancestral P-loop protein families which, according to Leipe & al. possibly harbor multiple activities, such as NTP hydrolysis and phosphate transfer<sup>410</sup>, the question of whether the real physiological function of this family is an ATPase arose.

At the same time, several other groups working on BYks reported an interesting phenomenon where their protein kinases (*B. subtilis* YwqD<sup>476</sup> and *E. coli* Wzc<sup>477</sup>) exhibited a weak ATPase activity. It was also the case observed in heat-shock Hsp70 protein family around 1990's<sup>103,478</sup> where, for instance *E. coli* DnaK which functions as a molecular chaperone and possesses an auto-kinase activity and a weak ATPase activity. It was proposed that ATPase might be an opportunistic activity of these protein kinases in the absence of their substrate partners.



**Figure 24:** Auto-kinase activity and Phosphotransferase activity of YdiB

Focus on *B. subtilis* YdiB, our preliminary results have confirmed the absence of GTPase activity in this enzyme<sup>10</sup>. It remains therefore the question of kinase activity. In the presence of  $\gamma^{32}$ -ATP and Mg<sup>2+</sup>, YdiB is indeed capable of autophosphorylating, as shown in the first lane of *fig. 24*.

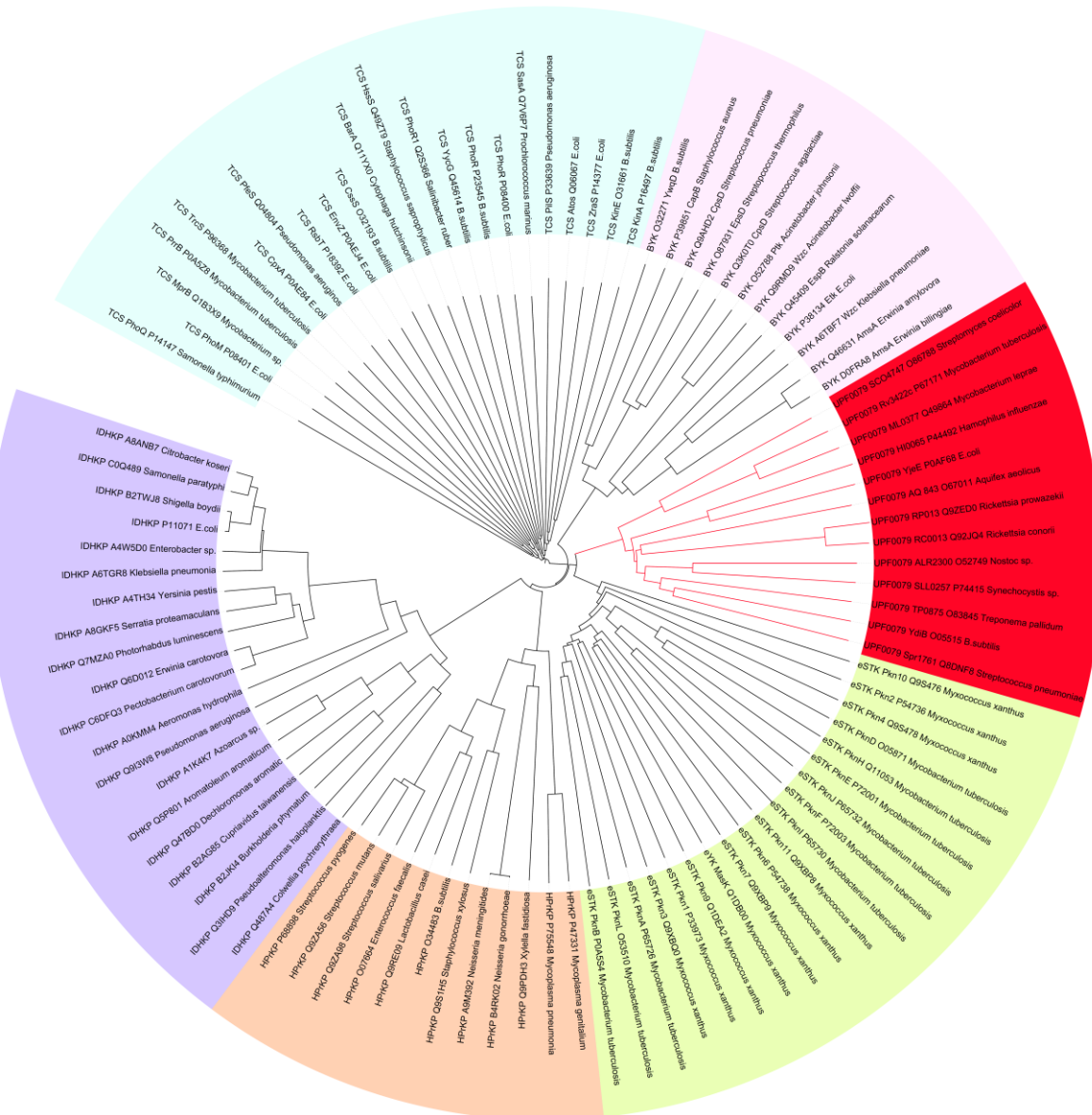
In order to verify if YdiB is able to transfer the phosphate group onto other proteins, two archetypes *in vitro* protein kinase substrates, the MBP (i.e. Myelin Basic Protein) and the Histone-H1 were separately put into contact with  $\gamma^{32}$ -ATP and Mg<sup>2+</sup> in the absence of YdiB (lane 2 and 4) or in the presence of YdiB (lane 3 and 5). Interesting results were obtained since these two substrates not only are phosphorylated by YdiB, but also increase significantly the signals corresponding to YdiB autophosphorylation. The latter remark will be further explored in the II.2.3.

The results obtained here clearly confirmed the protein kinase activity residing in YdiB, suggesting its implication in some phosphorylation cascade(s) of signal transduction.

### II.1.4. Evidence for a new class of protein kinases

Obviously, *B. subtilis* YdiB possess protein kinase activity. The same results were also obtained with *E. coli* YjeE and *Streptococcus pneumoniae* Sp1761 by our collaborators in Grangeasse's group at the Institute of Biology and Chemistry of Proteins, Lyon. The next question is to localize these protein kinases in the protein kinases' world. Given the functional genomic results that the "YjeE-like" family fell into uncharacterized protein families, and structural analysis brought into light a unique fold among Walker A loop-containing family, one can expect that UPF0079 would establish a new separate branch of protein kinases.

Well-known prokaryotic protein kinase families up to date comprise 5 major classical branches: Histidine kinases in Two-component systems, IDHK/Ps and HPrK/Ps discovered in the 1980's, eukaryotic Ser/Thr kinases of Hank's type in the 1990's; and the recently discovered branch of BYKs in the 2000's. At least 12 representatives of each class were collected from UniProtKB<sup>4</sup> in order to compare with UPF0079's protein kinase family revealed in the 2010's by our group. A total of 112 protein kinases were aligned by ClustalX<sup>479</sup> and phylogenetic tree was constructed using the Neighbor Joining method. 10000 bootstrap trials were performed for the phylogenetic analysis. Finally the phylogenetic tree was submitted to iTOL program<sup>471,472</sup> for visualization.



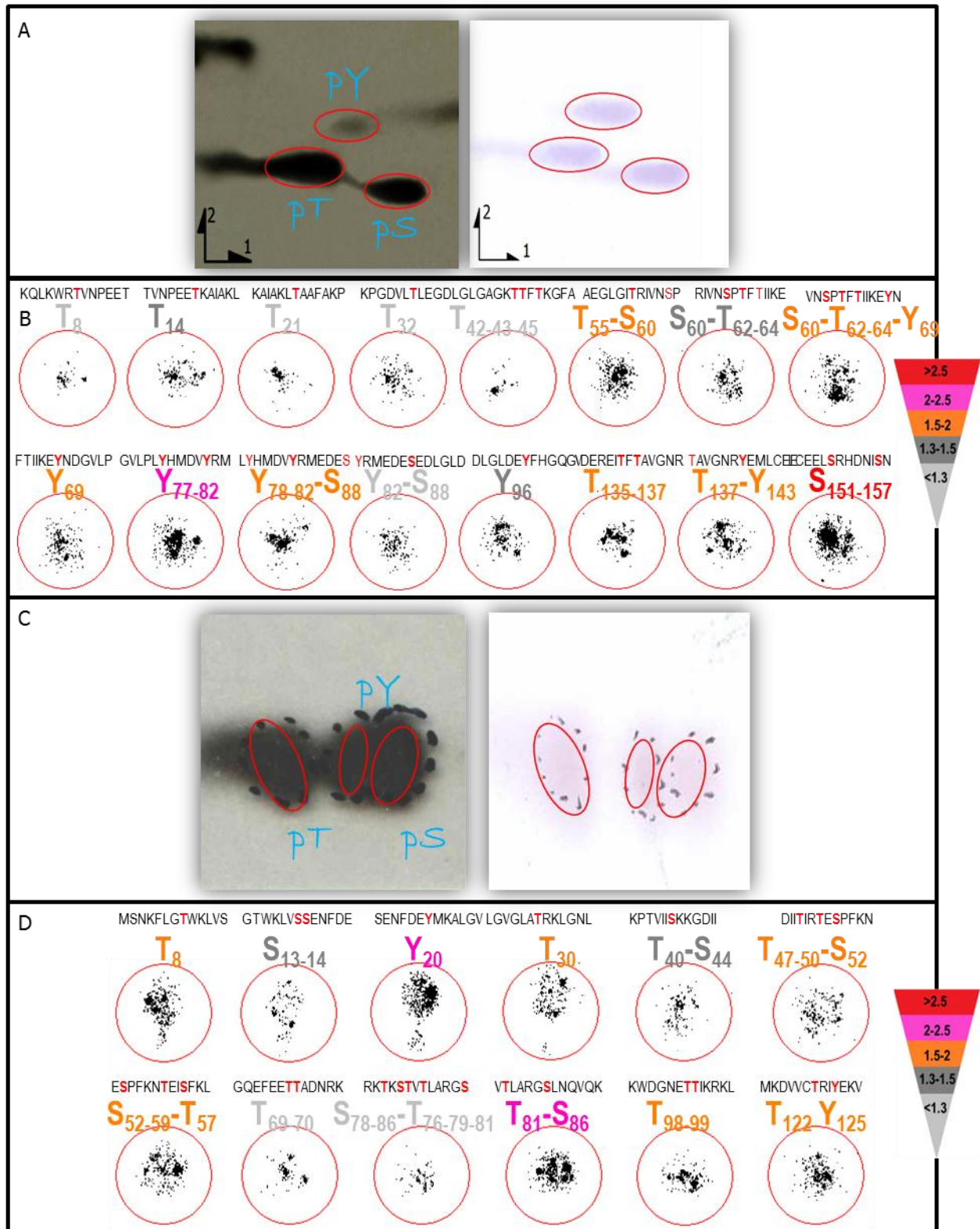
**Figure 25:** Phylogenetic analysis reveals new branch of bacterial protein kinases

Data collection was described in detail in the Material-Methods. The pale blue fan regroups Histidine kinases from different TCS systems. The pink one comprises of BYKs. The yellow-green fan presents eSTK of Hank's type. The orange one contains HPrK/Ps. The violet fan covers IDHK/Ps. The red fan is the new branch containing UPF0079 family-Ubks.

The phylogenetic tree presented in *fig. 25* clearly confirmed the existence of six separate branches corresponding to six different families of bacterial protein kinases. Beside the five previously mentioned families, UPF0079 family was found to establish the last separate branch (in red). This latter shares no similarity to other protein kinase families, except for the Walker A-loop as in the cases of HPrK/P and BYK. Given the fact that this new family is broadly conserved in bacterial kingdom, we proposed to name it Ubk for "Ubiquitous bacterial kinase".

## II.2. Enzymatic characterization of YdiB's kinase activity

## II.2.1. Phosphorylation site analysis



**Figure 26:** *In vitro* phospho-amino acid analysis of YdiB and MBP

A: Phospho-amino acid of autophosphorylated YdiB revealed by bidimensional electrophoresis

B: YdiB phosphosite analysis: Different 13 mer biotides containing potential phosphosites of YdiB were incubated with YdiB in presence of  $\gamma$ - $^{32}$ -ATP in kinase buffer at 30°C. Termination buffer was added after 5 hours of incubation and the reaction mixture was then spotted onto SAM2 Biotin Capture Membrane (Promega®). After extensive washing to remove unbound reaction components, quantification of signals corresponding to bound and labeled peptides was achieved using PhosphoImager (PerkinElmer®) and Gel-Pro Analyser software (Media Cybernetics®). Classification of quantified signals from weak (ratio signal/background < 1.3) to very strong (ratio > 2.5) was presented as color code bar in the right.

C: Phospho-amino acid of MBP phosphorylated by YdiB revealed by bidimensional electrophoresis

D: MBP phosphosite analysis: The same protocol as B was used with different 13 mer biotides containing potential phosphosites of MBP.

In order to identify the nature of phosphoamino acids that have been formed by covalent binding of phosphoryl groups, phosphorylated YdiB and MBP were separately subjected to acid hydrolysis followed by two-dimensional TLC. Autophosphorylated YdiB contained  $P_i$ -Thr,  $P_i$ -Ser and to a lesser extent  $P_i$ -Tyr (fig. 26A). YdiB clearly phosphorylated Ser, Thr and Tyr residues on MBP (fig. 26C).

Attempts to identify the phosphosites of YdiB and its protein substrates by mass spectrometry were not successful. The level of (auto)phosphorylation and therefore the ratio of phosphorylated/non-phosphorylated forms thus was not sufficient to be detected by mass spectrometry. Alternative approach using biotinylated peptides provided insights into potential phosphosites. Multisites of different natures, whether Thr, Ser or Tyr, either in YdiB (fig. 26B) or in MBP (fig. 26D) were found to be possibly phosphorylated.

It is still hard to point out the exact phosphosites since the interaction between short peptides and protein might not represent what truly happened with the whole folded protein. However, together with classical TLC, this result corroborates the global image where YdiB autophosphorylates and phosphorylates other protein on Ser, Thr and Tyr residues. The same profiles were obtained with *E. coli* YjeE and *S. pneumoniae* Sp1761 by Grangeasse's group, supporting the evidence of new class of bacterial protein kinases. To our knowledge, up to date, there has been only one another example of bacterial dual-specificity protein kinase. It was the case of *Chlamydophila pneumoniae* PknD, a kinase which autophosphorylated on Thr and Tyr residues and phosphorylated its substrate on Ser and Tyr residues<sup>480</sup>. However, it is worth noting that no Tyr-kinase activity was observed for PknD from *Chlamydophila trachomatis*<sup>165</sup>. Dual-specificity kinase activity in bacteria was thus, before our study, still questionable.

In eukaryotes where phosphorylation event was studied earlier with much more biological data, examples of dual-specificity protein kinases are available but also limited. Two typical exemplars should be mentioned: MEK (so called MAPKK/ERKK, i.e. Mitogen-Activated Protein Kinase Kinase/Extracellular-signal-Regulated Kinase Kinase) and DYRK (i.e. Dual-specificity-Tyrosine-Regulated Kinase).

The former MAPKK/ERKK family (including MEK1 and MEK2) are dual-specificity protein kinases that phosphorylate the activation loop of the characteristic MAPK/ERK (including ERK1 and ERK2) on Tyr and Thr residues of the TxY motif. Both phosphorylations are required for full activation of the MAPK/ETK in order to further stimulate the downstream effectors, many of which are transcription factors<sup>481</sup>.

The latter DYRKs phosphorylate their substrates on Ser/Thr residues, but are themselves autophosphorylated on a critical Tyr residue in their activation loop of YxY motif. This autophosphorylation occurs before the kinase leaves the ribosome and seemingly is issued from intramolecular (*cis*) phosphorylation<sup>482,483</sup>. However, Dyrk1A is active even without this Tyr phosphorylation<sup>484</sup>. Dyrk1A located on human chromosome 21 and responsible for inactivating NFAT (i.e. Nuclear Factor of Activated T cells) to promote its export from the nucleus. A reduction in the activity of NFAT may well account for many of the symptoms associated with Down's syndrome<sup>485</sup>.

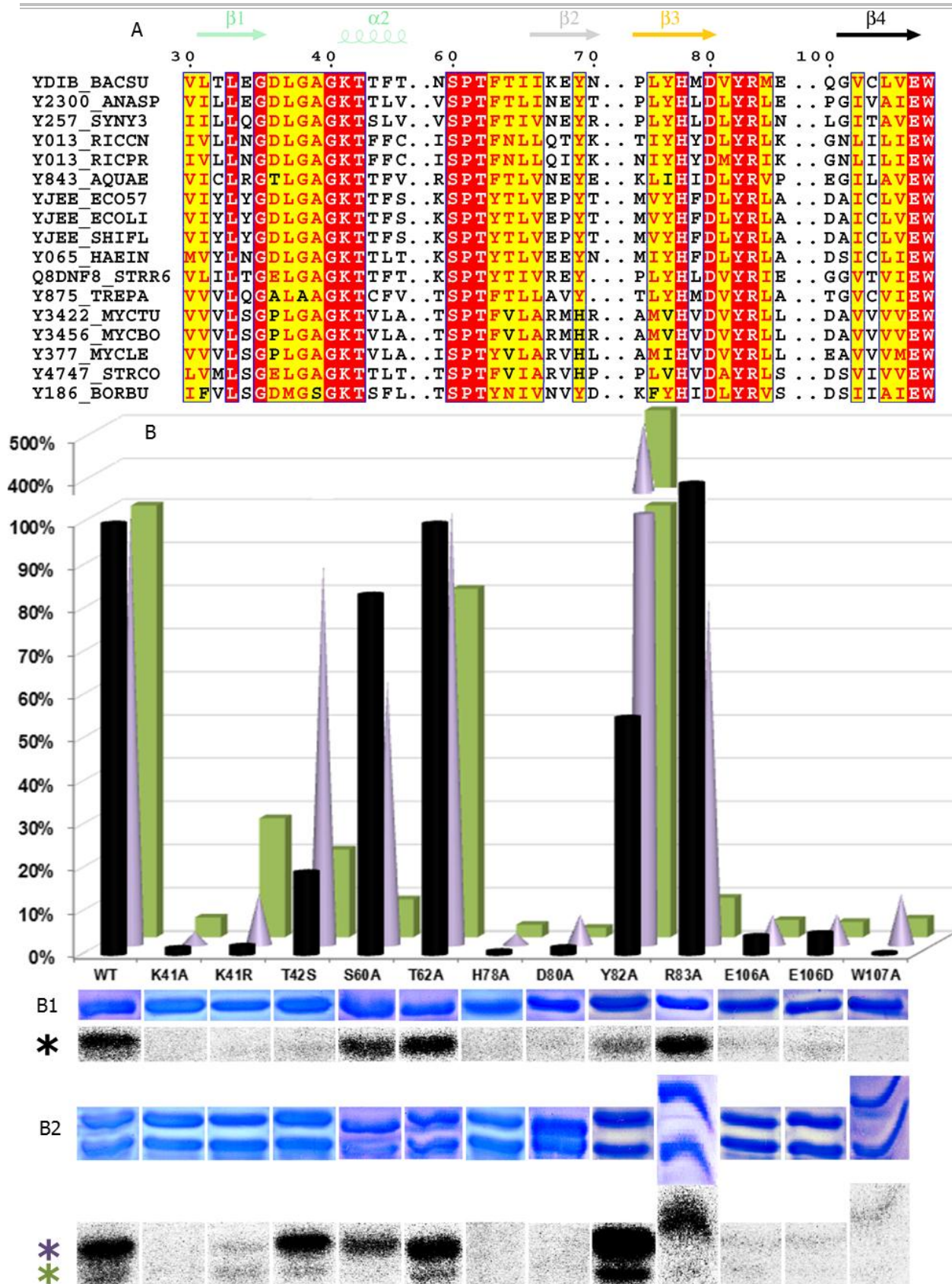
These eukaryotic dual-specificity protein kinases are overall derivatives from Ser/Thr kinases of Hanks' type with the additional capacity of (auto)phosphorylating on Tyr residues. The only case reported in bacteria, PknD from *C. pneumoniae* was also a eukaryotic-like Ser/Thr kinase. In conclusion, the Ubk-"YjeE-like" therefore is the first dual-specificity protein kinase family distinguishable from eSTK reported so far in both eukaryotic and prokaryotic kingdoms.

### II.2.2. Implication of conserved residues on kinase activity

As introduced in the II.1.1, UPF0079 is broadly conserved protein family. The alignment of 44 COG0802 proteins (fig. 21B) revealed some extremely conserved residues inside this family. In order to clarify implication of these residues on kinase activity of YdiB, 10 residues among the most conserved are muted one by one and their kinase activity were measured. The results are presented in the fig. 27 and the list of created mutants was the following: K41A, K41R, T42S, S60A, T62A, H78A, D80A, Y82A, R83A, E106A, E106D, W107A.

The crystal structure of *H. influenzae* YjeE will be used to pinpoint the lessons obtained from the structure-function relationship. By using the "Mutagenesis" function in Pymol software, we elucidated the local interactions around each mutated residues as following:





**Figure 27:** Implication of conserved residues on kinase activity of YdiB

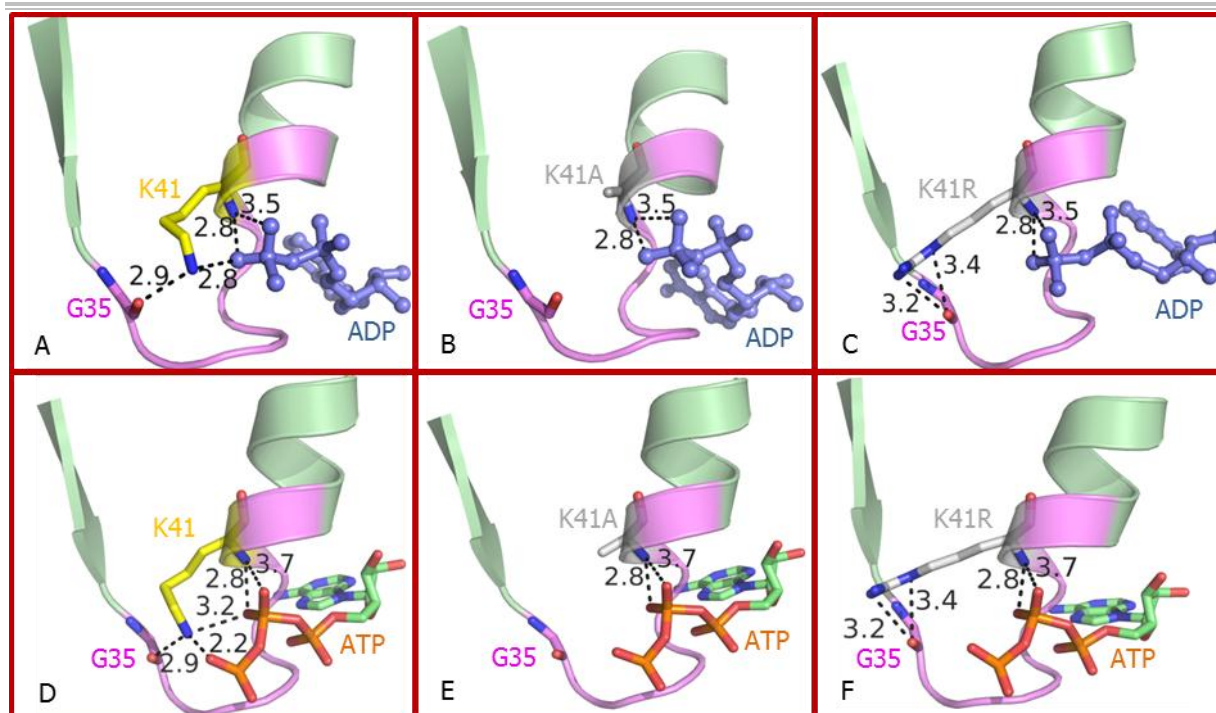
A: Extract of multiple sequence alignment from 17 UPF0079 homologs reveals conserved residues.

B: Effect of single mutation of each conserved residue on autokinase activity of YdiB and on its capacity of transferring the phosphate onto the MBP surrogate substrate.

B1: After incubation of native YdiB (WT) or YdiB mutants at 30°C for 15 minutes with  $\gamma^{32}$ -ATP and  $Mg^{2+}$ , the reactions were stopped by heating at 95°C then electrophoresis was carried out followed by coomassie blue staining (upper panels) and autoradiography (lower panels). The autokinase activities of WT and mutants were quantified by ImageJ and presented as black column. The signal corresponding to WT was chosen as 100%.

B2: As in B1 but in presence of MBP substrate. The violet cones and the green boxes correspond to autokinase activities of YdiB-WT and its mutants in presence of MBP substrate; and their kinase activities towards the MBP substrate, respectively.

*the lysine mutants of Walker A-motif, K41A and K41R*



**Figure 28:** Structural analysis focused on Walker A-lysine residue

- A: Interaction of K41 with ADP (3 potential contacts) and with the conserved G35 residue (1 potential contact).  
 B: Interaction of K41A with ADP (2 potential contacts). No potential contact between K41A and G35.  
 C: Interaction of K41R with ADP (2 potential contacts) and with the conserved G35 residue (2 potential contact).  
 D: Interaction of K41 with ATP (4 potential contacts) and with the conserved G35 residue (1 potential contact).  
 E: Interaction of K41A with ATP (2 potential contacts). No potential contact between K41A and G35.  
 F: Interaction of K41R with ATP (2 potential contacts) and with the conserved G35 residue (2 potential contact).

In 2004, Allali-Hassani in E. Brown's group<sup>9</sup> for the first time provided molecular information of the "YjeE-like" family on working with three Walker mutants of *E. coli* YjeE, K41A, T42A and D80Q. No appreciable difference in term of ATPase was observed between the WT and mutated proteins. This detected activity might thus correspond to the basal background ATPase observed in nucleotide-binding proteins in absence of their substrates or activators. In 2009, Karst in our group<sup>10</sup> revisited the ATPase activity of *B. subtilis* YdiB and confirmed the intrinsic ATPase of YdiB as this activity was absent in the mutant K41A.

In term of kinase activity, a strong abolishment; and a pronounced attenuation were observed when this Walker A-lysine was mutated into alanine or arginine respectively (*fig. 27-B, C-K41A, K41R*). We did a structural analysis to understand the molecular impact of this lysine on the function of YdiB.

As revealed in (*fig. 28-A*), K41 might have 3 contacts with the  $\beta$ -phosphate group of ADP and one contact with the conserved glycine 35 in order to keep the Walker-A loop in a proper conformation. When this lysine was mutated to alanine, it lost the ability to contact with the G35 residue and might therefore destabilize the catalytic loop. However, this latter interaction was possibly re-established in case where the arginine residue was introduced. In both cases, K41A and K41R, the ADP was always retained by at least 2 interactions. This explained why the *E. coli* YjeE possessed a strong affinity for ADP as reported in 2004<sup>9</sup> and by our preliminary results on *B. subtilis* YdiB (results not shown).

The fact that K41R also lost its kinase activity having kept the ability to interact with G35 and ADP suggests the participation of other type of interactions. Using the "Pair Fitting" function in Pymol, we introduced a molecule of ATP in place of ADP (*fig. 28, lower row*). The K41 indeed interacts with the phosphate  $\gamma$  of ATP (*fig. 28-D*). This interaction was lost in both cases, the K41A or K41R (*fig. 28-E, F*).

This analysis suggests that the lysine side chain is essential for the phosphotransfer reaction. Without the K41 residue, the rest of nucleotide is still tightly bound to the Walker A-loop but the phosphate  $\gamma$  is not correctly oriented, leading to the loss of kinase activity.

#### *the threonine mutant of Walker A-motif, T42S*

In 2009, Karst in our group measured the ATPase activity of T42S mutant, showing that this activity was attenuated when a low concentration of ATP was used, but it was not negligible in the presence of 5 mM ATP<sup>10</sup>. The result suggested the role of T42 in the affinity of the protein towards either ATP or Mg<sup>2+</sup>.

In term of kinase activity, when 10 nM of  $\gamma^{32}$ -ATP was used, the kinase activity of the T42S mutant was not as affected as in the case of K41A or K41R mutants, even so ~80% of activity was lost (*fig. 27B, black column and green box of T42S*). However, in presence of the MBP substrate, the autokinase activity was somehow restored (*fig. 27B, violet cone of T42S*).

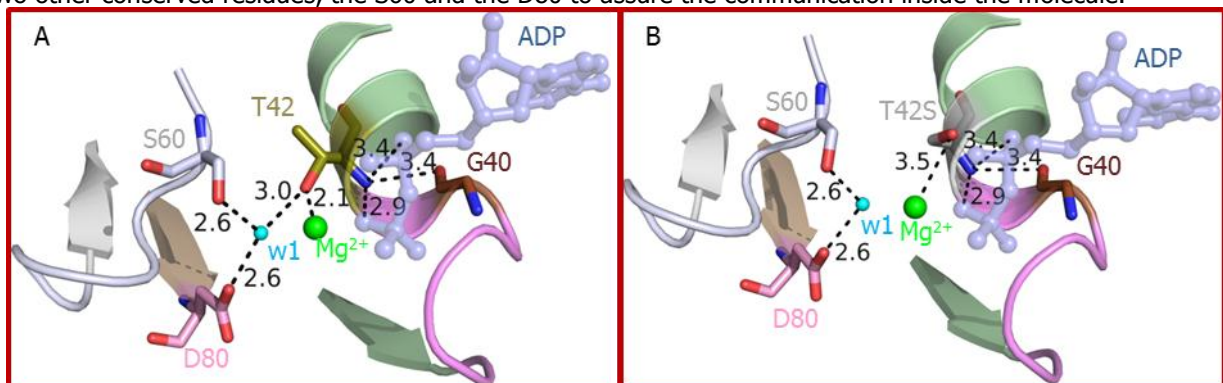
Looking inside the catalytic zone, we observed that this threonine played three different roles (*fig. 29A*):



The main chain of T42 interacts with the conserved G40 in order to secure the conformation of the helix  $\alpha_2$  vis-a-vis the Walker A-loop. Also this main chain participates in nucleotide binding by mean of two interactions with the phosphate  $\beta$  and  $\alpha$ .

The lateral chain of T42 is very close to the  $Mg^{2+}$  and thus obviously, together with the nucleotide they constitute two determinant factors which hold the cation in place.

Finally, through 2 hydrogen-bridges via a water molecule (named w1), the side chain of T42 interacts with two other conserved residues, the S60 and the D80 to assure the communication inside the molecule.



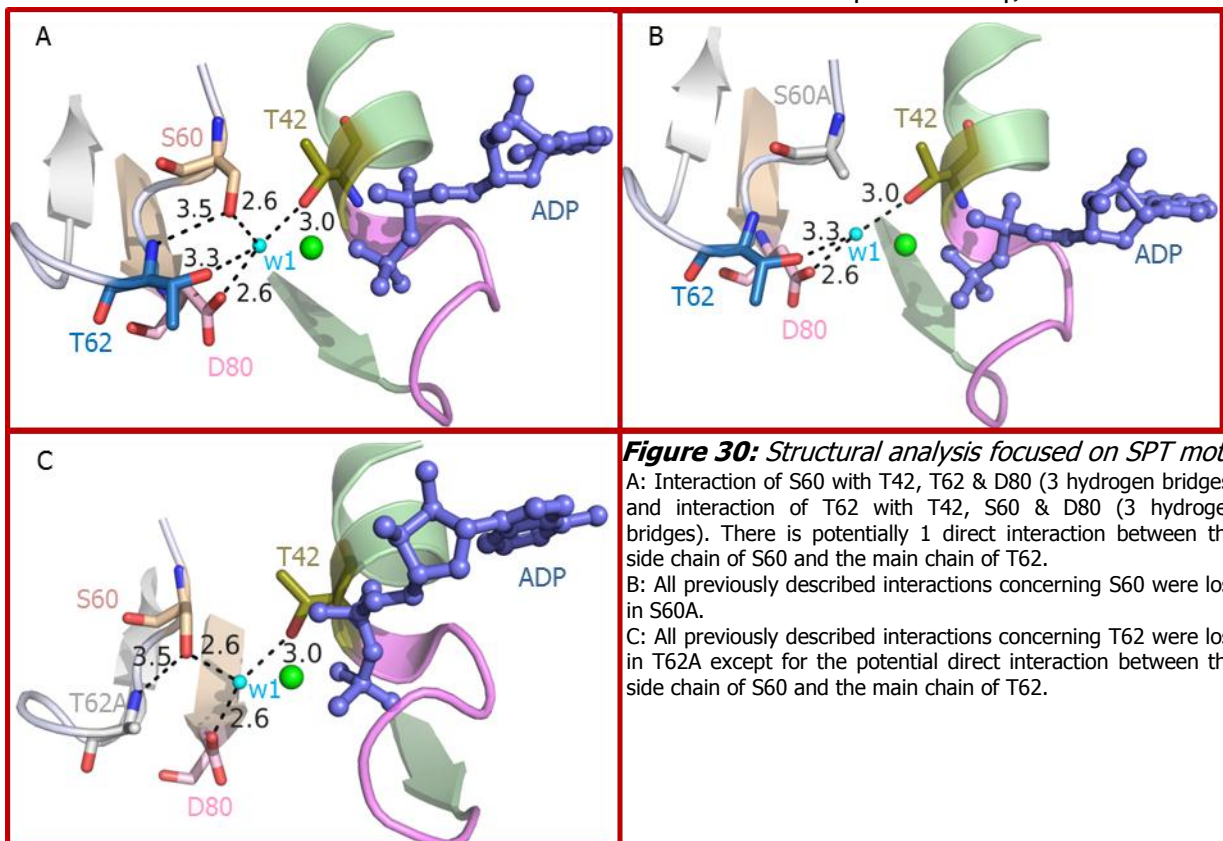
**Figure 29:** Structural analysis focused on Walker A-threonine residue

A: Interaction of T42 with ADP (2 potential contacts), with the conserved G40 residue (1 potential contact), with  $Mg^{2+}$ , with S60 and D80 through two hydrogen bridges (T42-w1-S60 and T42-w1-D80).

B: Interaction of T42S with ADP (2 potential contacts), with the conserved G40 residue (1 potential contact) and with  $Mg^{2+}$ .

When this threonine was mutated to serine (*fig. 29B*), the two hydrogen bridges were disconnected and the interaction to  $Mg^{2+}$  might be weakened due to increasing distance (from 2.1 to 3.5 Å). This analysis explained why the kinase activity of T42S mutant was strongly decreased but still not totally attenuated. If this seems to produces a major effect, one can imagine that in the presence of MBP substrate which triggers the conformational change, S60 and D80 might be brought closer to T42S and the connection would be restored.

*the mutants of SPT motif, S60A and T62A*



**Figure 30:** Structural analysis focused on SPT motif

A: Interaction of S60 with T42, T62 & D80 (3 hydrogen bridges) and interaction of T62 with T42, S60 & D80 (3 hydrogen bridges). There is potentially 1 direct interaction between the side chain of S60 and the main chain of T62.

B: All previously described interactions concerning S60 were lost in S60A.

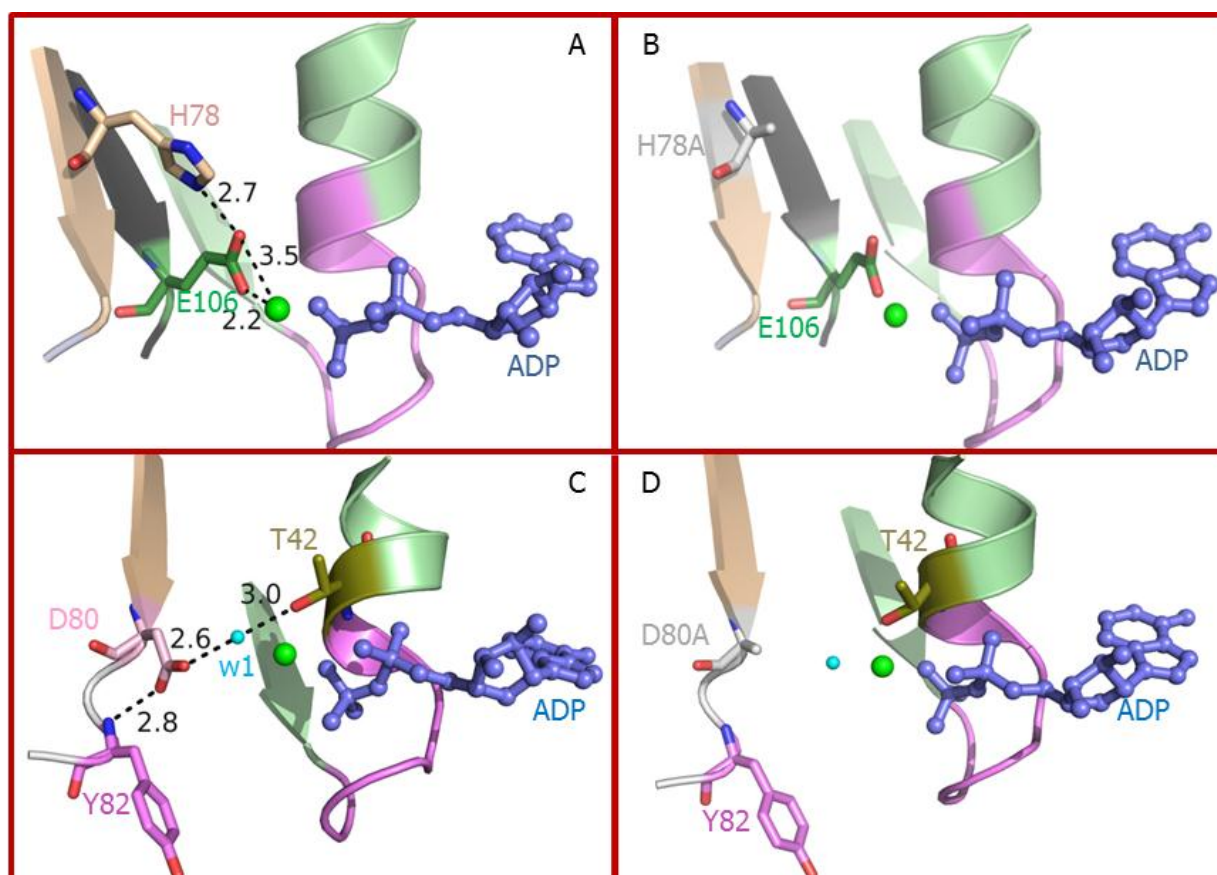
C: All previously described interactions concerning T62 were lost in T62A except for the potential direct interaction between the side chain of S60 and the main chain of T62.

In YjeE-like family, an invariant SPT motif is found close to the nucleotide-binding site. Despite the conservation of the motif, the mutation of S60 or T62 to alanine did not have strong effect on the autokinase activity of YdiB (*fig. 27B*, S60A and T62A, black columns and violet cones). This result suggests that SPT motif is not the principal autophosphorylation site of YdiB. In agreement with this argument, no strong signal was detected when the biotinylated peptide containing this motif (RIVNSPTFTIIKE) was incubated with YdiB in the presence of  $\gamma^{32}$ -ATP (*fig. 26B*).

Structural analysis focused on this motif revealed the participation of one active water named w1. This water is stably retained in its spatial position through many hydrogen bridges (*fig. 30A*) and also by interacting with  $Mg^{2+}$  to keep this cation in its ideal octahedral coordination (not shown here, *c.f. I.2.1 in Literature review*). Since the position of  $Mg^{2+}$  influences the orientation of nucleotide and therefore kinase activity, this w1 plays important role in kinase activity. With the fact that S60A and T62A still keep their autokinase activity, one can suppose that hydrogen bridges linked to S60 and T62 are not essential for the stability of w1. *Fig 30-B,C* indeed suggested that in each mutation, this water was still hold by at least 3 interactions even when the interaction with  $Mg^{2+}$  was not taken into account.

However, in the case of S60A, the phosphotransferase activity towards MBP substrate was strongly abrogated (*fig. 27B, S60A, green box*). This result suggested that in the presence of protein substrate, other factors than the stability of w1 need to be considered. It is worth noting that in eukaryotic protein kinases of the Hanks family, the conserved (S/T)P motifs have been also reported<sup>486,487</sup>. This motif is found within the critical P+1 loop, which is a point of contact between the kinase and its substrate<sup>23</sup>. The conserved S60 residue of the SPT motif in YjeE-like family therefore might concern substrate binding/recognizing capacity. This would explain why the phosphotransferase activity was attenuated in the case of S60A but not in T62A.

*the mutants of H78A and D80A*



**Figure 31:** Structural analysis focused on conserved histidine & glutamic acid

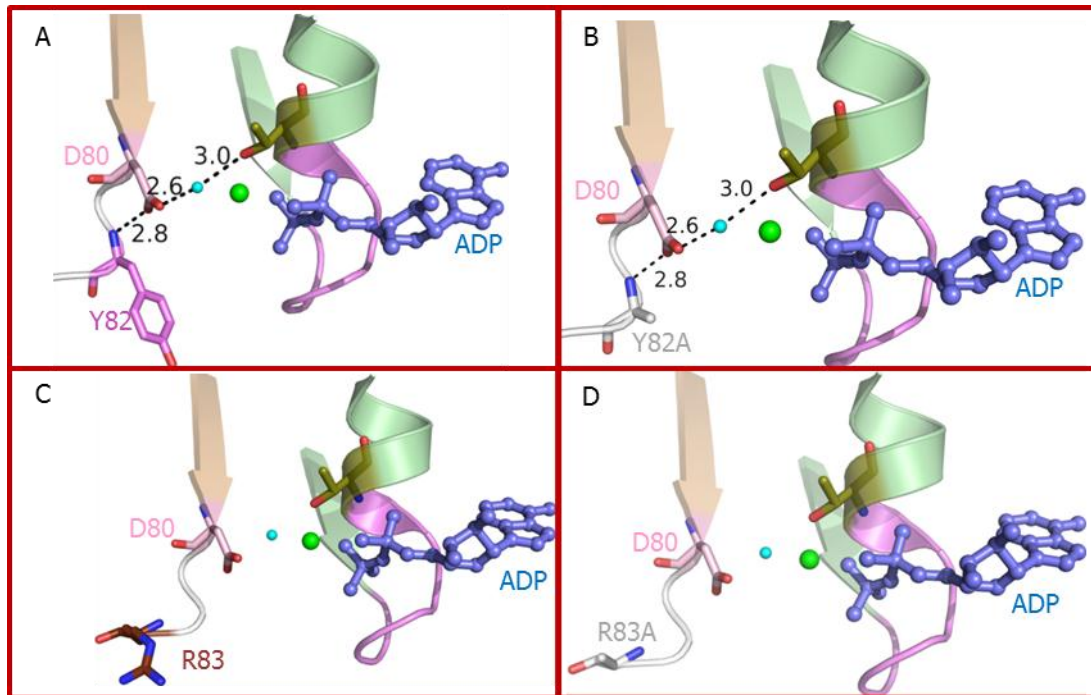
A: Interaction between H78 & E106 (1 potential contact). B: This interaction was lost on mutating H78 to alanine. C: Interactions between D80 & Y82 (1 potential contact) and D80 & T42 (1 potential water-bridge). D: These interactions were lost on mutating D80 to alanine.

The mutations of conserved H78 or D80 residues in alanine resulted in total abolishment of kinase activity of YdiB (*fig. 27B, H78A and D80A*). This might correspond to the loss of important contacts caused from the mutations.

In the case of H78, this residue directly contacts the conserved E106 of "hypothetical added strand" (*fig. 31-A, black strand, or c.f. II.1.2 for more details*). This interaction might be essential to orientate the E106 in right position to secure the co-ordination of  $Mg^{2+}$  as well as the proper conformation of ATP (*c.f. structural analysis of E106 for more details*).

In the case of D80, the side chain of this residue can interact directly with the main chain of Y82, securing the right fold of the ATP-binding site of YdiB; and also indirectly with the conserved T42 residue through a water-bridge involving the previously mentioned water w1. In contrast to the non-essential hydrogen bridges linked to S60 and T62 (as supposed in the precedent analysis of S60 and T62 residues), the bridge D80-w1-T42 seems to be essential for the stability of w1 and therefore essential for  $Mg^{2+}$  and nucleotide binding.

the mutants of conserved tyrosine and arginine residues, Y82A and R83A



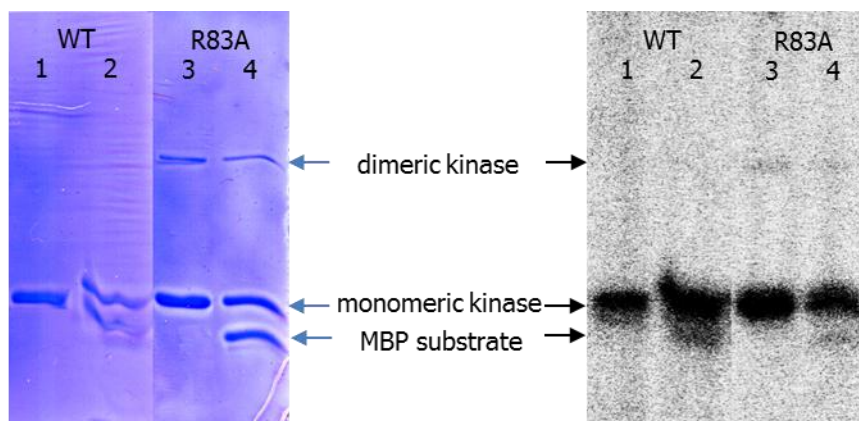
**Figure 32:** Structural analysis focused on conserved tyrosine and arginine residue

Interaction of the main chain of Y82 (A) or Y82A (B) with the side chain of D80. R83 (C) and R83A (D) have no potential interactions with side-chains of other residues.

When the conserved Y82 was mutated to alanine, about 40% of autokinase activity was lost (*fig. 27B*, Y82A, black column). However in the presence of MBP substrate, both autokinase and kinase activities of this mutant were strongly increased, to ~ 500% of magnitude in comparison with wild-type YdiB.

When the neighbor conserved R83 was mutated to alanine, the autokinase activity of R83A mutant were seemingly not affected, even more activated when the MBP substrate was absent (*fig. 27B*, R83A, black column and violet cone). However the phosphotransferase activity of this mutant was strongly attenuated. The phenomenon observed in this case was similar to the one obtained with S60A mutant.

Interestingly, in term of “theoretical” local interactions, upon mutation of Y82 or R83 to alanine, no influence was detected (*fig. 32-A, B, C, D*). Since Y82 and R83 are located in the surface zone exposed to solvent, one can suppose that these residues are implicated in substrate binding/recognition or in oligomerization of the protein kinase itself.



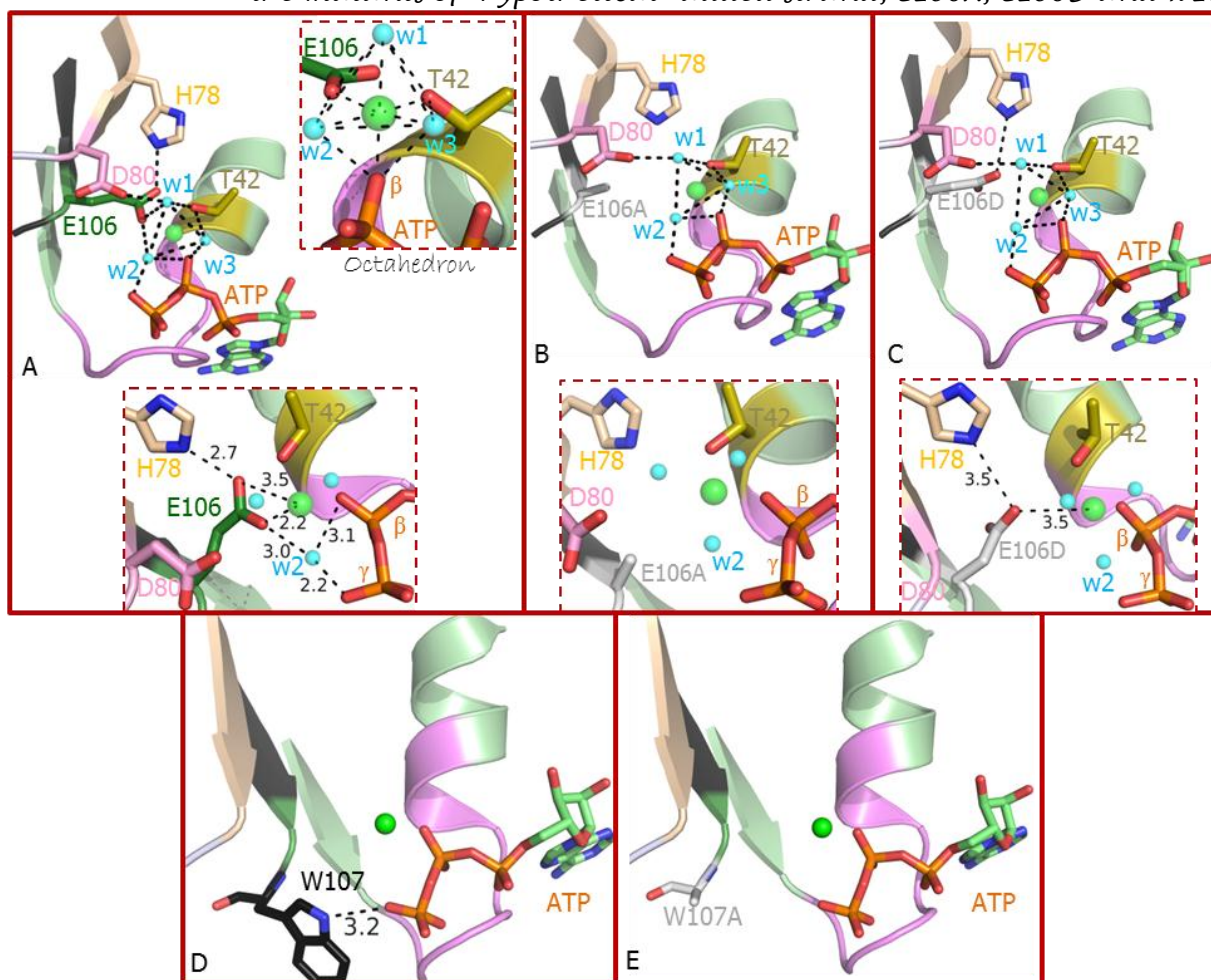
**Figure 33:** R83A mutant forms stable dimers

After incubation of YdiB-WT and YdiB-R83A mutant at 30°C for 15 minutes with  $Mg^{2+}$  and  $\gamma^{32}$ -ATP; in the presence (even lanes, 2 and 4) or absence (odd lanes, 1 and 3) of MBP substrate, the reactions were stopped by heating at 95°C during 15 minutes before electrophoresis followed by coomassie blue staining (left) and autoradiography (right).

In other previously characterized mutants, after being heated at 95°C during 15 minutes then loaded into denaturing SDS-PAGE gel, the oligomeric forms of YdiB (WT or mutants) are negligible. The quantitative comparison of signals corresponding to monomeric forms therefore is significantly enough. In some cases however these oligomeric forms are very resistant and thus are not negligible. It was the case of R83A mutant (*fig. 33*), where the mutation of R83 to alanine seemingly favors the oligomerization. Since the autokinase activity of this mutant was more important than the WT protein, one can assume that the oligomerization might activate this activity. The link between oligomerization and autokinase activity will be addressed latter, in II.2.4.



the mutants of "hypothetical" added strand, E106A, E106D and W107A



**Figure 34:** Structural analysis focused on glutamic acid and tryptophan in "hypothetical" added strand

The ATP molecule was introduced in the position of ADP using "Pair Fitting" function in Pymol software

A: Interaction of E106 with H78 (1 potential contact); with  $Mg^{2+}$  (2 potential contacts); with T42 (1 hydrogen-bond via w1) and with ATP (2 hydrogen-bonds via another water molecule named w2).

B: Loss of all above mentioned interactions in E106A mutant.

C: Loss of 3 hydrogen-bonds with ATP and T42 and weaker interactions with H78 and  $Mg^{2+}$  in E106D mutant.

D: Interaction between W107 and phosphate  $\gamma$  of ATP. This interaction was lost in case of W107A mutant (E).

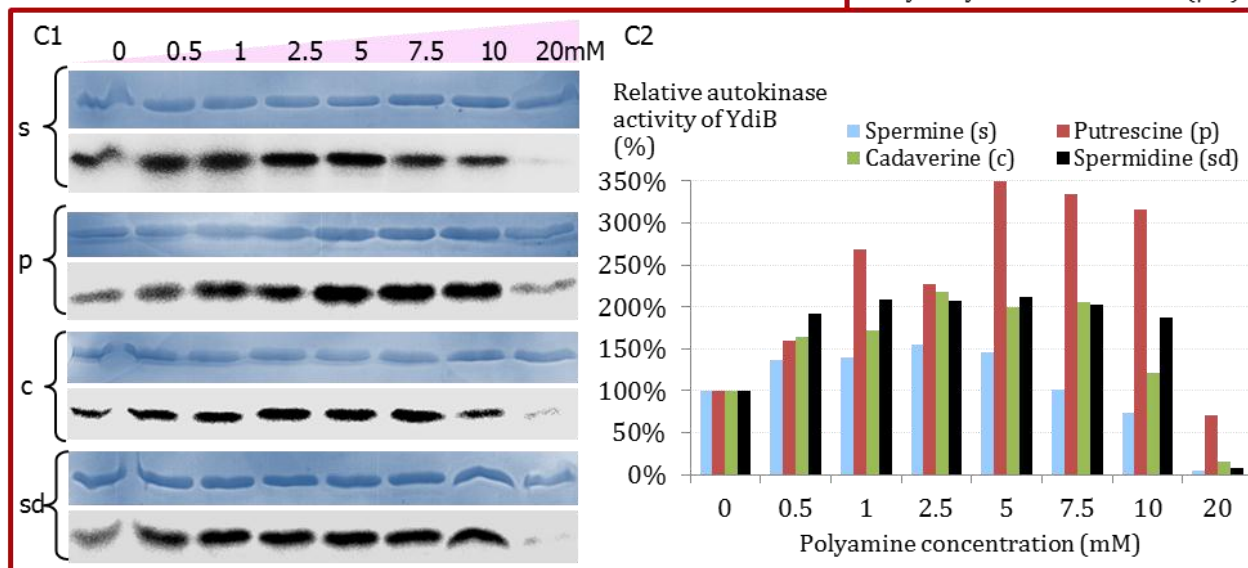
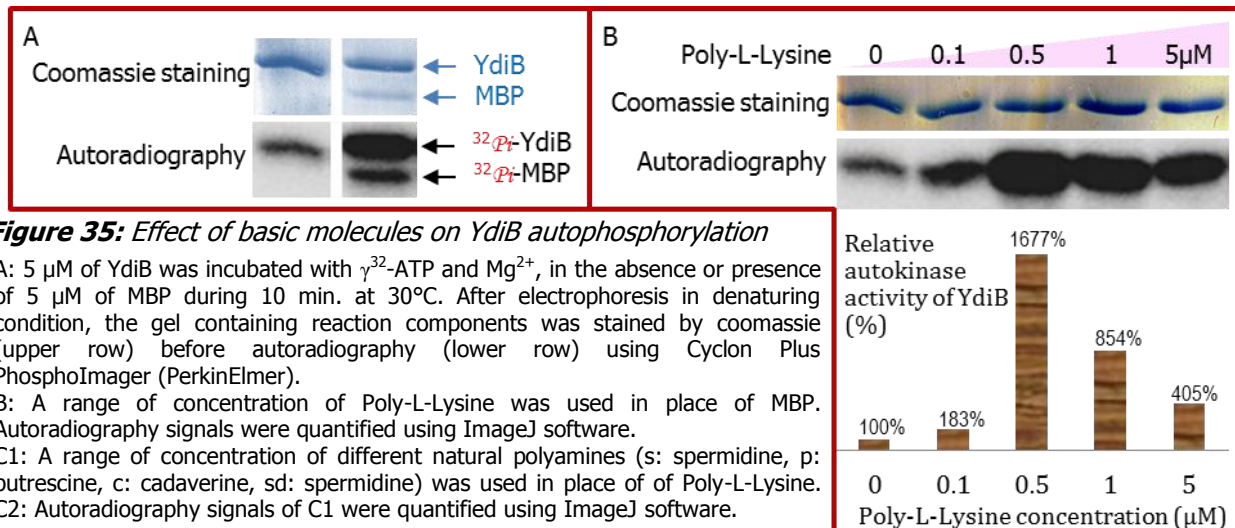
The conserved glutamic acid residue at the C-ter of a hydrophobic strand VCLVE106 has been still the subject of controversy: whether it belongs to the Walker B-strand of KG group or the added strand of ASCE group (*c.f.* II.1.2 for more explication). This special residue could interact with Walker A-T42 residue through a hydrogen-bond involving active water w1 (*fig.* 34 A, octahedron). It could also interact with  $\beta$  and  $\gamma$  phosphates of ATP via a water molecule called w2 as well as with  $Mg^{2+}$  via two direct interactions (*fig.* 34-A).

With numerous potential important contacts, it was not surprising that the mutation of this E106 residue to alanine (*fig.*27 B, E106A) fully abolished the kinase activity of the protein mutant. Structural analysis indeed confirmed the loss of all potential contacts of E106 in E106A mutant (*fig.* 34-B).

In order to clarify the real role of this E106, one needs to pinpoint the most important contact(s) for which it is responsible. The E106D mutant thus was constructed. Interactions of E106D with H78 and  $Mg^{2+}$ , even weaker than the native E106, were restored. However, this new mutant lost contacts with both ATP and T42 residue (*fig.* 34 C), thus did not restore the octahedral co-ordination of  $Mg^{2+}$ . Since the kinase activity of E106D mutant was also totally abrogated (*fig.*27 B, E106D), we could then confirm that the conserved D80 but not E106 belongs to the Walker B-motif. The conserved E106 meanwhile serves likely in an essential step in phosphotransfer mechanism involving in keeping  $Mg^{2+}$  in an ideal co-ordination and in keeping ATP in a proper conformation<sup>488-492</sup>.

Next to the conserved E106 at the extremity of the added strand is another conserved residue, W107. The mutation of this tryptophan to alanine also revoked kinase activity of the protein mutant (*fig.*27 B, W107A). Except for side-chain connection, this residue has only one potential electrostatic interaction with ATP. By withdrawing charge via interaction with the oxygen atom of phosphate  $\gamma$ , this W107 would contribute to increase the electrophilicity of the  $\gamma$ -phosphorous atom. Together with its neighbor E106, these residues could be essential factors initiating the transfer of phosphate  $\gamma$ .

## II.2.3. Effect of basic molecule on kinase activity



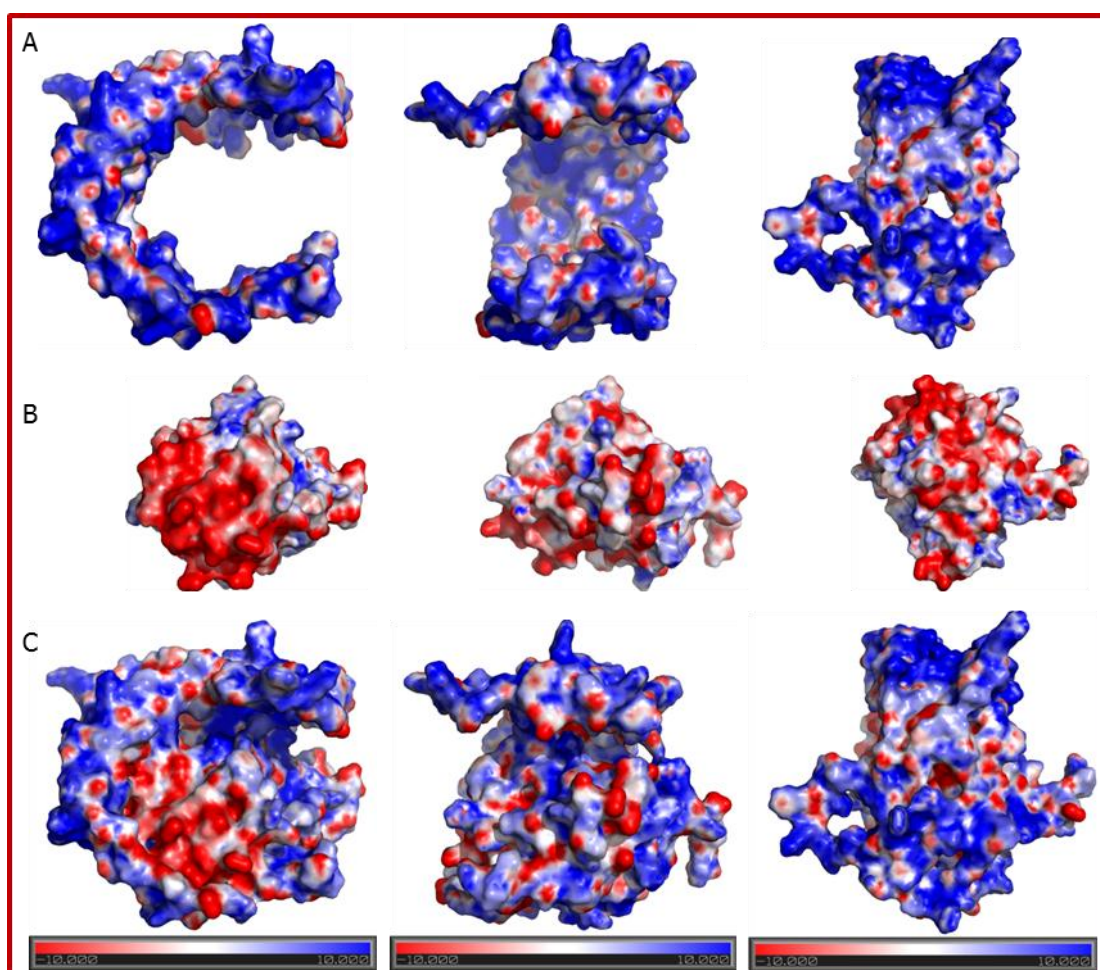
When YdiB was incubated with MBP (*i.e.* Myelin Basic Protein), we observed an interesting phenomenon where the signal corresponding to YdiB's autophosphorylation was much stronger than in the absence of MBP (*fig. 35A*). The same effect had been obtained with Histone H1 (*c.f. fig. 24*), which is also a very basic protein<sup>493–496</sup>. These results strongly suggested that somehow basic molecules could interact with YdiB and activate its kinase activity.

The activation of Tyr, Ser/Thr kinases by basic poly-amino acids and proteins containing basic amino acid domains has been extensively investigated in *Eukaryota* in the late of 1980s<sup>497–501, 502–505</sup>. In most cases, Poly-L-Lysine was found to be the most effective activator that increased the activity of eSTY kinases up to 2-10 times. Recently, the first example of activation of prokaryotic kinase by MBP and Poly-L-Lysine was reported in *Bacillus subtilis* PrkC by Absalon & al.<sup>161</sup>. However PrkC is also a eukaryotic-like Ser/Thr kinase that shares features with eSTYKs of Hanks' type, raising the question if basic molecule stimulation is specific to eukaryotic-like STY kinases. We answered this question showing that YdiB, a new type of bacterial kinase followed the basic molecule-activated mode, either with basic proteins (MBP and Histone H1) or basic poly-amino acid (Poly-L-Lysine) or natural polyamines (spermine, putrescine, cadaverine and spermidine). Natural polyamines stimulated the autophosphorylation of YdiB by up 2- to 3.5-fold at physiological concentrations of 0.5–20mM<sup>506</sup> (*fig. 35-C1, C2*), which is comparable with the activation of protein kinase A by spermine<sup>507</sup>. Whereas poly-L-lysine seemed still to be the best activator with the maximum stimulator capacity of more than 16-fold at molar ratio of kinase/activator of  $\sim 10:1$  (*fig. 35-B*). This ratio is similar to that obtained with previous studies on insulin receptor tyrosine kinase<sup>499</sup> or eukaryotic-like Ser/Thr kinase PrkC<sup>161</sup>. To our knowledge, after PrkC, YdiB presents the second example of basic molecule-activated kinase in prokaryote.

Dose-response patterns of either Poly-L-Lysine and natural polyamines for autophosphorylation of YdiB are biphasic. Depending on the nature of polyamines, beyond some concentrations, the effect became negative for autophosphorylation (*fig. 35-C2*). Interestingly, similar effects were reported with insulin receptor tyrosine kinase autophosphorylation<sup>499</sup> as well as the phosphorylation of its substrate, calmodulin<sup>498</sup>. Explanation for this biphasic phenomenon might be found on considering three different following hypotheses:



The first hypothesis aiming to explain the basic-molecule activated mechanism were suggested for the first time by Fujita-Yamaguchi & al.<sup>499</sup> who pointed out plausible direct interaction between Poly-L-Lysine and insulin-receptor tyrosine kinase. Later, Leroy & al.<sup>508-510</sup> provided the model of spermine-binding CK2 (*i.e.* casein kinase 2, a eukaryotic Ser/Thr kinase), where ionic interaction between positive charges of spermine and negative charges of acidic amino acids of CK2 provoked conformational change in CK2, thus facilitating the phosphorylation of its substrate. In case of YdiB, using the model of YdiB (*c.f.* II.1.2) and MBP (1qcl.pdb) as well as the available Propka 3.0 server<sup>511</sup>, we predicted the global charge of YdiB and MBP at pH 7.4 around -10 and +20 respectively, suggesting strong ionic interaction between MBP and YdiB. Using available [Pdb2pqr server](#)<sup>512</sup> for Poisson-Boltzmann electrostatics calculations and [Pymol 1.3 software](#) for the visualization, the resulting electrostatic potentials of YdiB and MBP models were presented in *fig. 36-A, B*. In agreement with predicted global charges, the surface of MBP is dominated by blue color corresponding to positive charges, whereas YdiB is covered by red color of negative charges. By using [Pathdoc beta 1.3 server](#)<sup>513,514</sup>, several candidate-complexes kinase-substrate YdiB-MBP were established. The best docking solution in term of *Geometric shape complementarity score* was chosen for calculating of electrostatic potentials as described previously and presented in *fig. 36C*. These analyses consolidate the hypothesis of strong ionic interaction between YdiB and basic molecules including MBP and Histone H1 proteins as well as natural polyamines and Poly-L-Lysine. Upon such interaction, YdiB might be subject of conformational change which promote the transfer of phosphate inside the enzyme and also possibly to its potential substrates.



**Figure 36:** Electrostatic potentials of models of MBP, YdiB and complex kinase-substrate YdiB-MBP

A:  $\pm 10$  kT/e electrostatic potential of MBP model (1qcl.pdb) in PyMOLplotted on the solvent-excluded surface.

B:  $\pm 10$  kT/e electrostatic potential of YdiB model (*c.f.* II.1.2) in PyMOLplotted on the solvent-excluded surface.

C:  $\pm 10$  kT/e electrostatic potential of YdiB-MBP complex model in PyMOLplotted on the solvent-excluded surface.

The second hypothesis provided first by Günther & al.<sup>515</sup> and later by Meksuriyen & al.<sup>507</sup> focused on the formation of a complex containing Polyamine,  $Mg^{2+}$  and ATP. Polyamines interact weakly with ATP alone and more efficiency with  $\gamma$  and  $\beta$  phosphates of  $Mg^{2+}$ -bound ATP. Given that  $Mg^{2+}$  also interacts with ATP via  $\gamma$  and  $\beta$  phosphates, it was proposed that polyamines compete with  $Mg^{2+}$  for binding to ATP. However this competition is not *stricto sensu* since the binding sites of  $Mg^{2+}$  and spermidine on ATP only partially overlap. The ternary complex of ATP- $Mg^{2+}$ -spermine thus truly exists. At neutral pH, adenine base existed in equilibrium between *anti* and *syn*-conformation with respect to the ribose moiety of ATP alone, but shifted to the *anti*-form in binary ATP- $Mg^{2+}$ -complex. This shift might be essential for the recognition of ATP at the active site of enzyme, thus explaining the indispensable role of divalent cations in nucleotide-binding protein. In ternary ATP- $Mg^{2+}$ -spermine complex, adenine is still in *anti*-conformation, but the presence of spermine provoked conformational change of  $\gamma$  and  $\beta$  phosphates of ATP<sup>507</sup>, which might trigger a series of events leading to the transfer of  $\gamma$

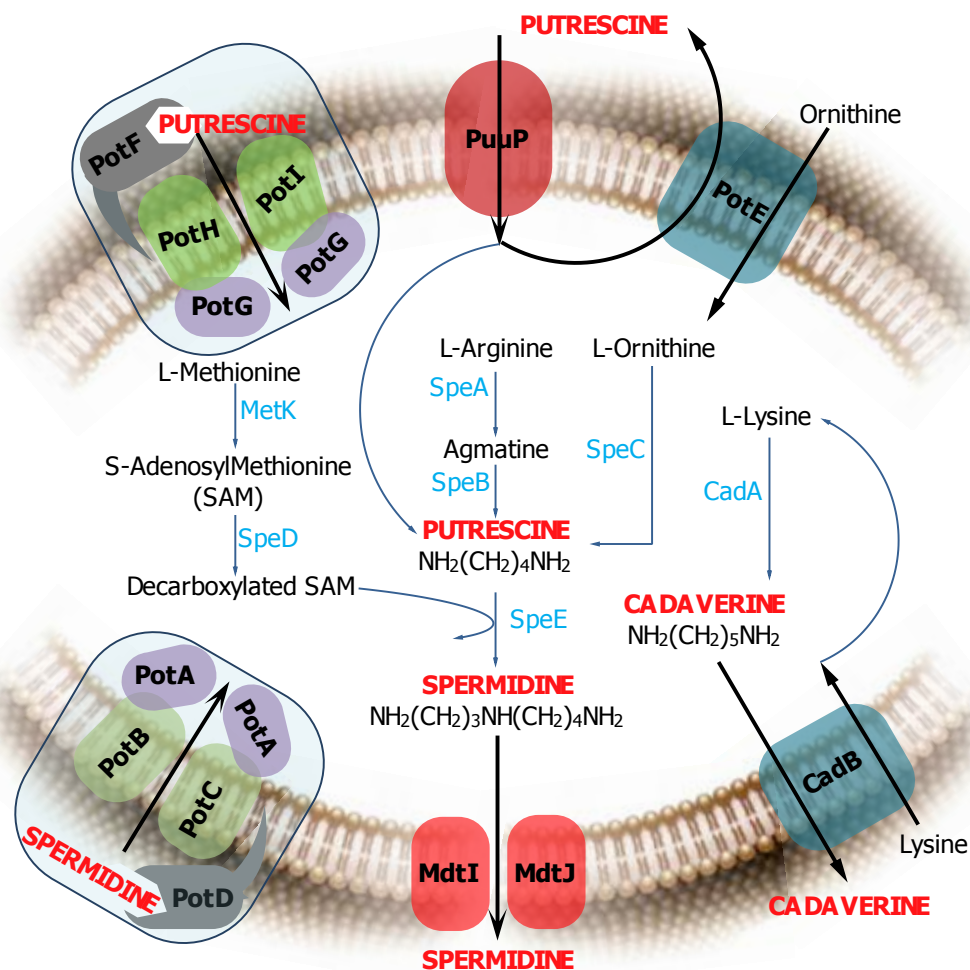


phosphates. However, at high concentration of polyamine, the binding of  $Mg^{2+}$  to ATP is actually inhibited, releasing free  $Mg^{2+}$  and binary ATP-polyamine complex.

The last hypothesis contributing to explain the biphasic effect of basic molecules might include their "side effect" of aggregation. In 1985, Heffetz & al.<sup>516</sup> for the first time reported that binding of anti-receptor antibodies to insulin receptor tyrosine kinase triggered receptor aggregation, which was also necessary for its kinase activity. In 1989, Kohanski & al.<sup>500</sup> and Fujita-Yamaguchi & al.<sup>499</sup> separately described that polycations including poly-L-lysine promoted aggregation of insulin receptor tyrosine kinase by binding to acidic domain of the receptor. In the case of YdiB, when the molar ratio of YdiB/MBP or YdiB/Poly-L-Lysine was kept constant but either in more concentrated working-solution or with bigger working-volume, we indeed observed the formation of precipitates. It thus was plausible that at small working-volume (20  $\mu$ L), the aggregation of YdiB by high concentration of basic molecules although not visible actually occurred, leading to the loss of autokinase activity.

...what's the physiological link between YdiB and natural polyamines?...

All previously mentioned models and hypotheses somehow explained the mechanism by which basic molecule contact and activate kinase activity of YdiB. If the results obtained *in vitro* truly reflected what happened *in vivo*, given that almost all *in vivo* events are not fortuitous but well calculated, one can expect that by following *in vivo* traces of natural polyamines, we could obtain more information concerning YdiB.



**Figure 37:** Polyamines in prokaryote: biosynthesis, uptake and export

At physiological pH, there are two polyamine uptake systems; both of them belong to ABC transporter family (*i.e.* ATP-Binding Cassette transporters). PotABCD system prefers spermidine, and PotFGHI system is putrescine specific<sup>517</sup>. Two other antiporters, PotE for putrescine/ornithine and CadB for cadaverine/lysine exchange are respectively in the same operon with genes for SpeF (ornithine decarboxylase) and CadA (lysine decarboxylase)<sup>518,519</sup>. Two new identified uniporters are PuuP for putrescine uptake involving in a new putrescine utilization pathway and MdtIJ for spermidine extrusion when spermidine is overaccumulated. Putrescine is synthesized by two different pathways from ornithine or arginine. Prokaryotic cells share common mechanism for spermidine synthesis from putrescine as in eukaryote, but in contrast, no mechanism for spermine was found in prokaryote in normal condition. However, in *speABC* knock-out mutant of *E. coli*, spermine was synthesized in place of spermidine and putrescine, suggesting that spermine biosynthesis occurs only when other polyamines are not available<sup>506</sup>.

Studies of polyamines in prokaryote, even less abundant than that in mammalian cells, are also available. Biosynthesis, uptake and export of putrescine, spermidine and cadaverine are resumed in the *fig. 37*.

Irrespective of mammalian or bacterial cells, polyamines can interact with RNA, nucleotide triphosphates, protein, RNA and ribosome. ~60-90% of each polyamine, either putrescine, spermidine, spermine or cadaverine, are found in complexes with RNA<sup>520</sup> to simulate protein synthesis<sup>521,522</sup>. Polyamines also induce *in*

---

*in vivo* assembly of 30S ribosomal subunits<sup>523-526</sup>. Binding of polyamines to ribosomes resulted in increasing of accuracy of codon usage during protein synthesis<sup>527-529</sup>, of +1 ribosomal frameshift efficiency<sup>530,531</sup> and facilitating translational read-through of mRNAs with the stop codon UAA<sup>530</sup>.

In the next section (*c.f.* II.3.2), we will find *in vivo* co-localization between YdiB and ribosome as well as phosphorylation of different ribosome-associated proteins by YdiB only in the presence of polyamines or poly-L-lysine. Interaction between polyamines and YdiB therefore will make sense in reinforcing the suggestion that YdiB might be linked to ribosome.

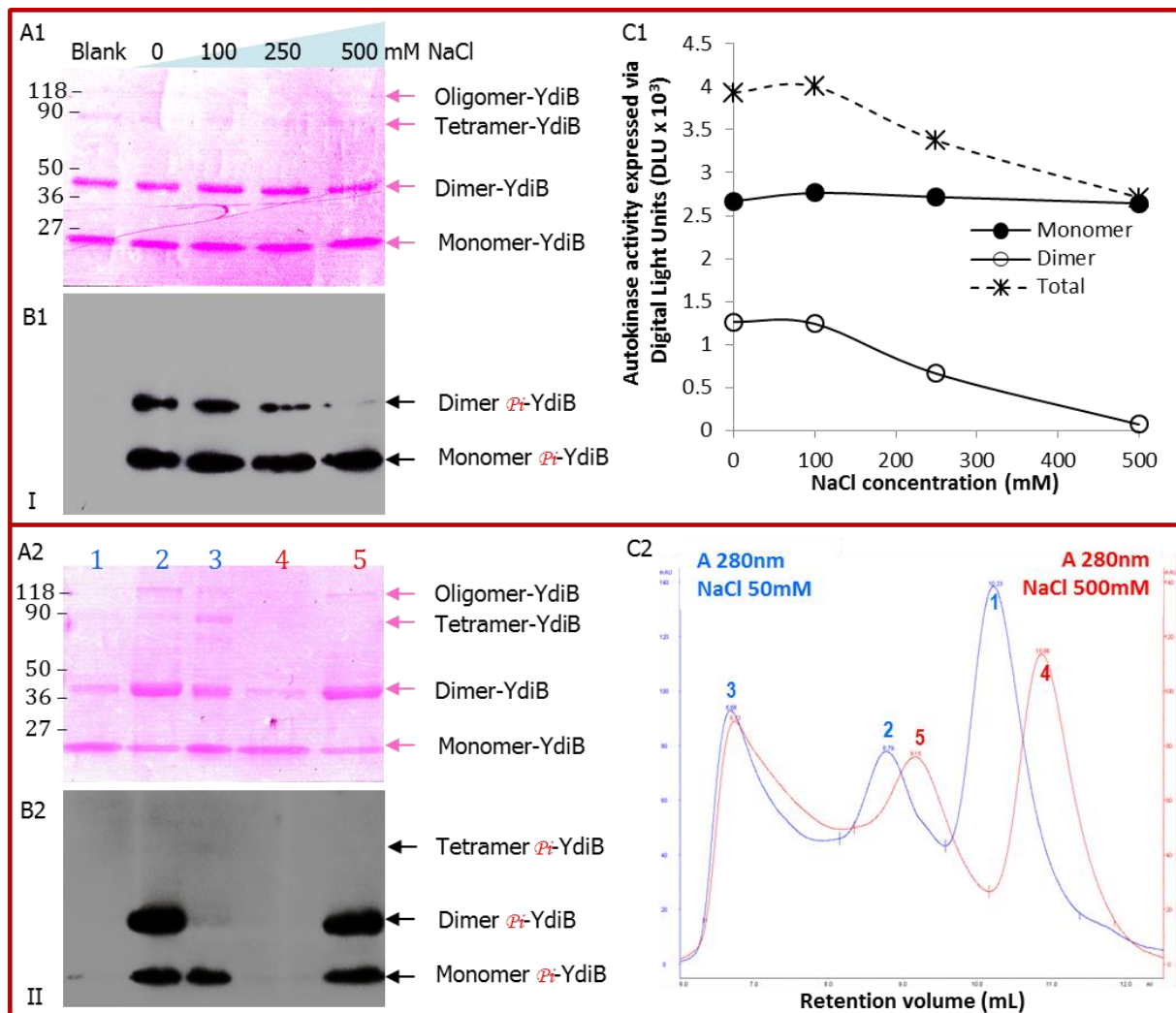
Not only playing important role in protein synthesis and/or in the synthesis and function of ribosome, polyamines also participate in responding to physiological stress. When cells are exposed to oxidative stress, reactive oxygen species provoke base modifications in high GC content DNA sequences and breakage of double-strand. The question of cellular survival requires overproducing of adaptive protection tools, e.g. superoxide dismutase (SOD) responsible for protecting nucleic acids from the damaging effects of superoxide radicals<sup>532</sup>. Spermidine and spermine as free radical scavengers conjunctionally function with SOD to reduce DNA breakage by oxygen radicals<sup>533-535</sup>. *E. coli speABCDE* knock-out mutant leading to putrecine and spermidine-deficient cells could not survive in oxidative stress conditions that are otherwise non-toxic to wild-type cells<sup>536</sup>. Polyamines therefore plausibly play role in regulation of toxic effects coming from oxidative stress.

In the section II.3.3, evidence for the importance of YdiB in protecting cells from toxic effects of paraquat-an oxidative stress inducer; as well as activation of Sod activity via phosphorylation by YdiB will be addressed. Once again, interaction between polyamines and YdiB therefore will make sense in pointing out the implication of YdiB in oxidative stress regulation.

Finally, another coincidence that needs to be taken into account concerns the subcellular localization of YdiB and polyamines. We previously showed that YdiB is located at the periphery and at the poles of *B. subtilis*<sup>10</sup>. As for polyamines, the discovery of different uptake and extrusion systems proved their circulation at the level of cell membrane<sup>517,537</sup> (*fig. 37*) and thus, potentially could interact with YdiB.

Alternatively, because of their unique charge-structure conformation, polyamines could be sandwiched between two negatively charged layers<sup>506</sup>. Since polyamines are able to interact with phospholipids<sup>538,539</sup>, they can serve as electrostatic bridges between lipid bilayer membrane and YdiB. One interesting example supports to this hypothesis is the case of MBP which bind to the SH3-domain called Fyn of tyrosine kinase Scr and tether this domain to the phospholipid membrane<sup>540,541</sup>. The signal transduction processes which occur at the cell surface level are well studied in eukaryote. For instance, three components (Raf, MEK and ERK) of the phosphorylation cascade ERK (Extracellular-signal-Regulated Kinase) are held in place at the cell surface by the scaffolding protein KSR1. The latter is the suppressor of Ras1 protein kinase responsible for initiating the ERK phosphorylation cascade<sup>481</sup>.

## II.2.4. Effect of oligomerization on kinase activity



**Figure 38: Oligomerization and autokinase activity of YdiB**

I: Dose-response of NaCl for autophosphorylation of YdiB: After incubation of YdiB with 250  $\mu$ M of ATP $\gamma$ S and different concentration of NaCl at 30°C during 10 min., the reaction mixtures were subjected to alkylation by 1.6 mM final of PNBM (*i.e.* p-nitrobenzyl mesylate) at room temperature for 2h. After electrophoresis and PDVF membrane blotting, Ponceau Red was used to reveal blotted proteins (A1). Thiophosphoproteins was recognized by Thiophosphate Ester Specific Rabbit Monoclonal antibody and the latter was then recognized by rabbit anti-IgG horseradish peroxidase antibody. Revelation of YdiB autophosphorylation was achieved by autoradiography using chemiluminescence (B1) and signal was quantified by ImageJ software (C1). The blank lane with 100 mM NaCl but without ATP $\gamma$ S gave no signal, confirming the specificity of the method.

II: Autokinase activity of Oligomeric, Dimeric and Monomeric YdiB: Different fractions corresponding to Oligomeric (n°3), dimeric (n°2 & n°5) and monomeric forms (n°1 & 4) were collected from two different size-exclusion chromatographies (blue: NaCl 50 mM and red: NaCl 500 mM) (C2). 8  $\mu$ g of protein in each fraction were subjected to western-blot as described in I. Total blotted protein was stained by Ponceau Red (A2) and thiophosphoprotein was revealed by autoradiography (B2).

In 2009, Karst & al. in our group showed that both *B. subtilis* YdiB and *E. coli* YjeE are capable of forming oligomers *in vitro* and *in vivo*. The ATPase activity of YdiB was regulated by oligomerization and the latter is modulated by salt concentration. More precisely, high concentration of salt induced oligomer disassembly into monomeric forms which is more active in term of ATPase<sup>10</sup>. Since oligomers were detected *in vivo* and that ATPase activity was not favored by oligomerization, one can anticipate that ATPase is not the principal physiological function of YdiB *in vivo*.

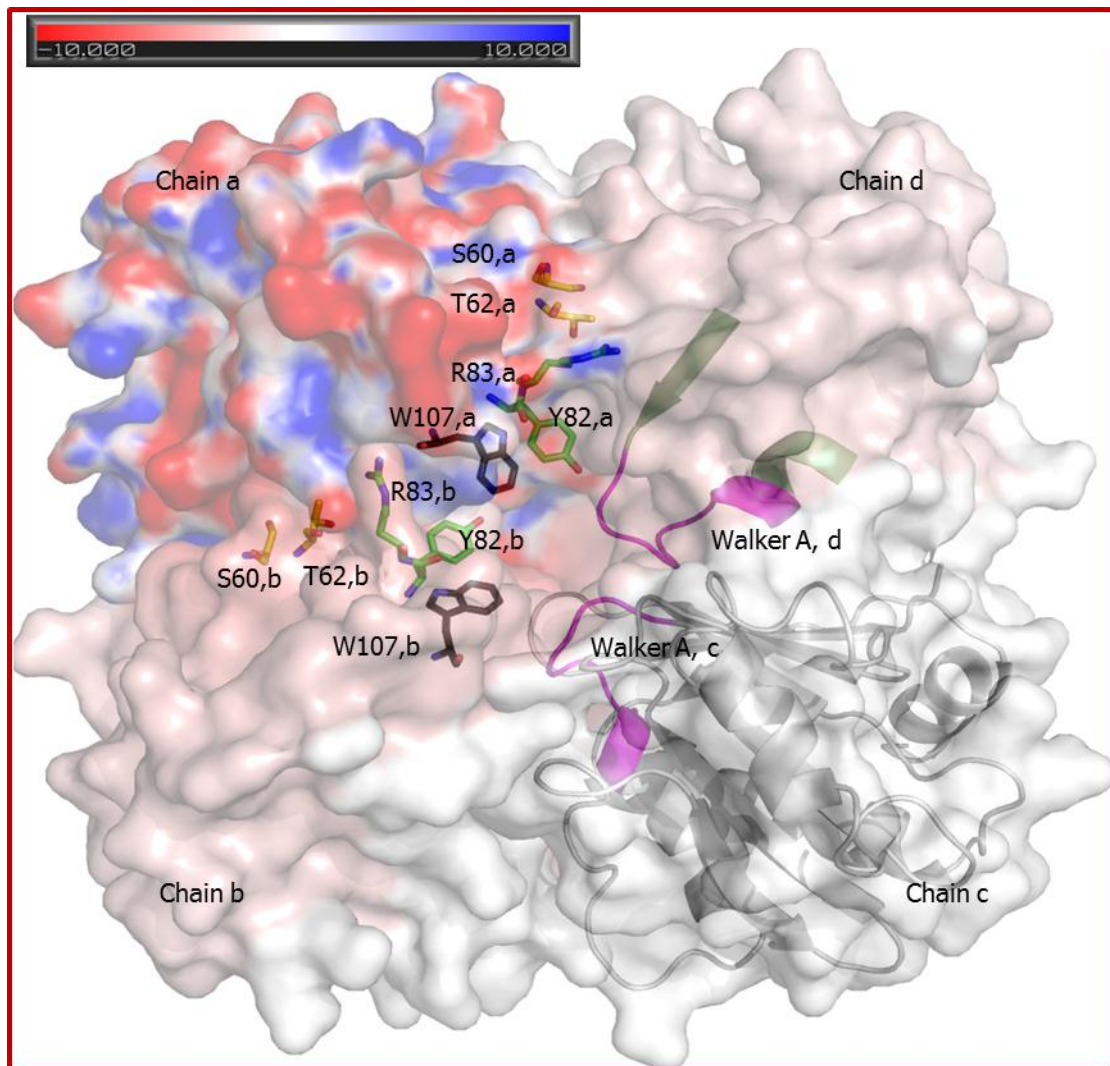
In line with this preliminary result, we then investigated influence of salt concentration and thus oligomerization of YdiB on its kinase activity. When increasing NaCl concentrations were used, although no big difference in term of oligomer/monomer ratio was observed after SDS-PAGE electrophoresis (*fig.* 38-A1) the autokinase activity actually changed (*fig.* 38-B1). Signal quantification using ImageJ software indeed confirmed that while autokinase activity of monomeric forms remained constant, the dimeric form was seemingly less phosphorylated, resulting in diminution of total autokinase activity with increasing salt concentrations (*fig.* 38-C1). This result suggested that high ionic strength disfavored kinase activity. Alternatively, if low ionic strength truly induced oligomerization (which was unfortunately not detected by electrophoresis in denaturing conditions as 10 minutes of reaction were not enough for stable oligomer formation), one can expect that kinase activity depends on oligomerization which is itself modulated by ion-strength.

In order to clarify the second hypothesis, purified YdiB was injected into size-exclusion chromatography at low (50 mM of NaCl) and high ion-strength (500 mM of NaCl). The chromatogram indeed confirmed the tendency of YdiB to form oligomers at low salt concentration as previously described<sup>10</sup> (*fig.* 38-C2). Please keep

in mind that each chromatographic peak corresponds not only to one form of YdiB but to a mixture of different forms with a majority of monomer (called monomer fraction) or dimer (called dimer fraction), etc... At the exit of the chromatography column, 8  $\mu\text{g}$  of protein from different fractions corresponding to monomer, dimer and high-molecular-weight oligomer were collected and subjected to autokinase assay using ATP- $\gamma$ -S as phosphate donor. The blotted membrane colored with Ponceau-R effectively reflected the distribution of different species in each collected fraction. Interestingly, monomeric forms revealed almost no kinase activity (fig. 38-B2, lane 1 & 4), whereas dimeric fractions seemingly possess all kinase activity of YdiB (fig.38-B2, lane 2 & 5). The partial loss of activity in higher oligomeric fractions might be due to some aggregation (fig. 38-B2, lane 3). This result obviously confirmed that oligomerization favors kinase activity of YdiB and this activity therefore is strongly suspected to be the principal physiological function of YdiB *in vivo*.

...which residues are implicated in oligomerization ? ...

Given that oligomerization is essential for YdiB to exhibit its kinase activity, finding the residues that are implicated in oligomerization will certainly give insight into molecular mechanism of this activity. If the implicated residues fall into conserved residues which have been previously described in II.2.2, the concerning mechanism will be representative for the whole YjeE-like family.

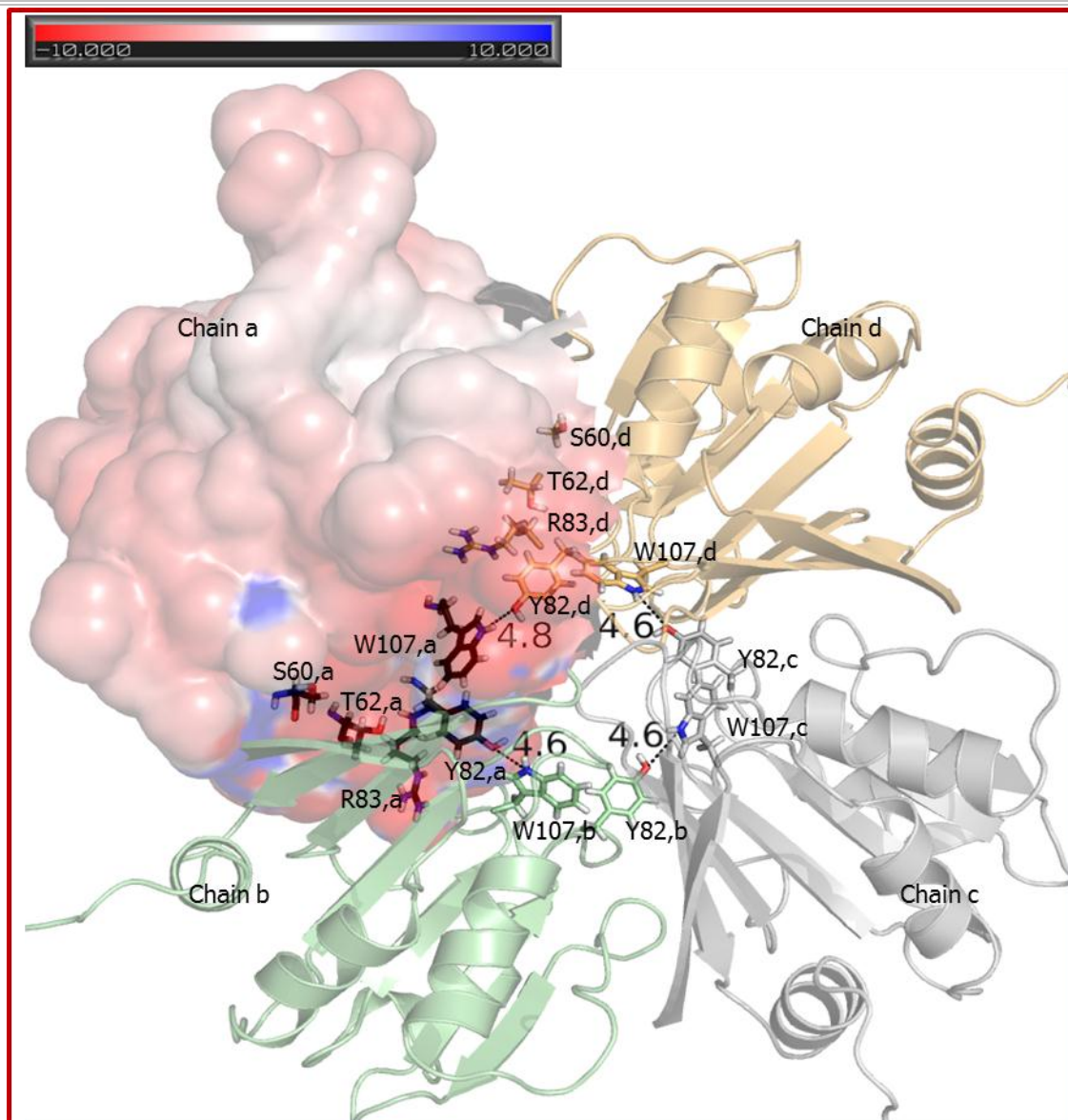


**Figure 39:** Model of *H. influenzae* YjeE tetramer and implication of conserved residues on oligomerization

The structure of tetrameric YjeE was constructed based on 1f9.pdb, chain A and Symmdock server version beta 1.0<sup>513,542</sup>. Chain A was presented with  $\pm 10$  kT/e electrostatic potential surface calculated via Pdb2pqr server version 1.7.1<sup>512</sup>. Chain C was presented as cartoon inside transparent surface. Walker A motifs of chain C and D were presented as magentas cartoon loops. Conserved residues possibly implicated in oligomerization were showed as sticks and numbered according to *B. subtilis* YdiB's sequence.

Evidence for *in vivo* tetramer formation was obtained with both *B. subtilis* YdiB and *E. coli* YjeE<sup>10</sup>. By using the available crystal structure of monomeric YjeE<sup>8</sup> (1f9.pdb) as well as the Symmdock beta 1.0 server<sup>513,542</sup>, several candidate-homotetramers of YjeE were established. The best docking solution in term of *Geometric shape complementarity score* was chosen for calculating electrostatic potentials via Pdb2pqr 1.7.1 server<sup>512</sup>. Pymol 1.3 software was used for visualization and analysis of oligomerization involving residues (fig. 39). Interestingly, according to InterProSurf server<sup>543</sup>, of the total 44 residues located in inter-subunit interfaces, five conserved residues S60, T62, Y82, R83 and W107 were found (YdiB sequence numbering, *c.f.* II.2.2). The same result was obtained with the model of homotetrameric YdiB (fig. 40) based on the model of YdiB monomer (*c.f.* II.1.2).





**Figure 40:** Model of *B. subtilis* YdiB tetramer and implication of conserved residues in oligomerization

A possible tetrameric YdiB was constructed based on the model of YdiB (*c.f.* II.1.2) and Symmdock server version beta 1.0<sup>513,542</sup>. Chain A was presented as solvent-accessible surface of  $\pm 10$  kT/e. Electrostatic potential calculated via Pdb2pqr server version 1.7.1<sup>512</sup>. Chain B, C, D were presented as cartoon. Conserved residues present in subunit-interfaces were showed as sticks. Potential electrostatic contacts between W107 and Y82 of different chains were presented as black dashes with calculated distance in Å°.

It is noteworthy to recall that interactions set up the subunit-interfaces are not based on solid covalent bonds, but on the accumulation of a set of weak liaisons that can unite two polypeptide chains. One subunit-interface is characterized by the nature and affinity of the interactions comprising that interface. Whatever type of interactions, whether Van der Waals, hydrogen, disulfide, or ionic bonds, are generated by only two types of interfacial hydrophobic or polar residues. Fundamental aspect of protein complex recognition and formation relies on *Electrostatic complementarity* and *Geometrical shape complementarity*, which are in turn assured by electrostatic interactions and hydrophobic effects.

Interfacial polar residues not only participate in Van der Waals interactions and electrostatic salt-bonds, but also engage in hydrogen-bridges and interactions via intermediate water molecules usually located in cavities of subunit-interface. These interactions contribute not only in oligomer stabilization, but also determine interfacial specificity<sup>544</sup>. Interfacial hydrophobic residues tend to cluster together to minimize their contacts with water. On folding, their side chains are buried within the oligomer, releasing water molecules. This hydrophobic effect increases the entropy of the system, making favorable contribution to the free energy of the folding, thus stabilizing the oligomer<sup>545</sup>.

Returning to the case of tetrameric YjeE and YdiB models, S60, T62 and R83 indeed could make electrostatic salt-bridges with several residues of adjacent subunit but none of these latter are conserved residues. This analysis thus does not allow revealing a general oligomerization mechanism. However, please keep in mind that interface formation is the result of accumulating many weak liaisons. Interfacial interactions involving S60, T62, and R83 therefore, although might be not essential, should be taken into account. Mutations of S60 or R83 in alanine did not alter autokinase activity but strongly decreased their phosphate-transferase activities towards MBP (*c.f.* II.2.2), suggesting the multifunction of these residues, not only in stabilizing oligomerization, but also in substrate-recognition/binding.



Y82 and W107 are also conserved residues present in the putative interacting surface. These residues, in the model of tetrameric YdiB and YjeE, are located in the inter-interface to form a compact core of four nucleotide-binding loops surrounded by four Tyr and four Trp. Aromatic side-chains of both residues, excluding the polar OH of Tyr and NE of Trp, are hydrophobic. The models therefore make sense, given that hydrophobic residues tend to gather to make a tightly packed core on extruding water. Mutations of W107 to alanine dramatically altered autokinase and protein phosphate-transferase activities of YdiB (*c.f.* II.2.2, *fig.* 27B). W107 was proposed to be engaged in increasing the electrophilicity of the  $\gamma$ -phosphorous atom of nucleotide (*c.f.* II.2.2, *fig.* 34). Here we suggest that its hydrophobic effect might be involved in stabilizing oligomers, thereby modulating kinase activity.

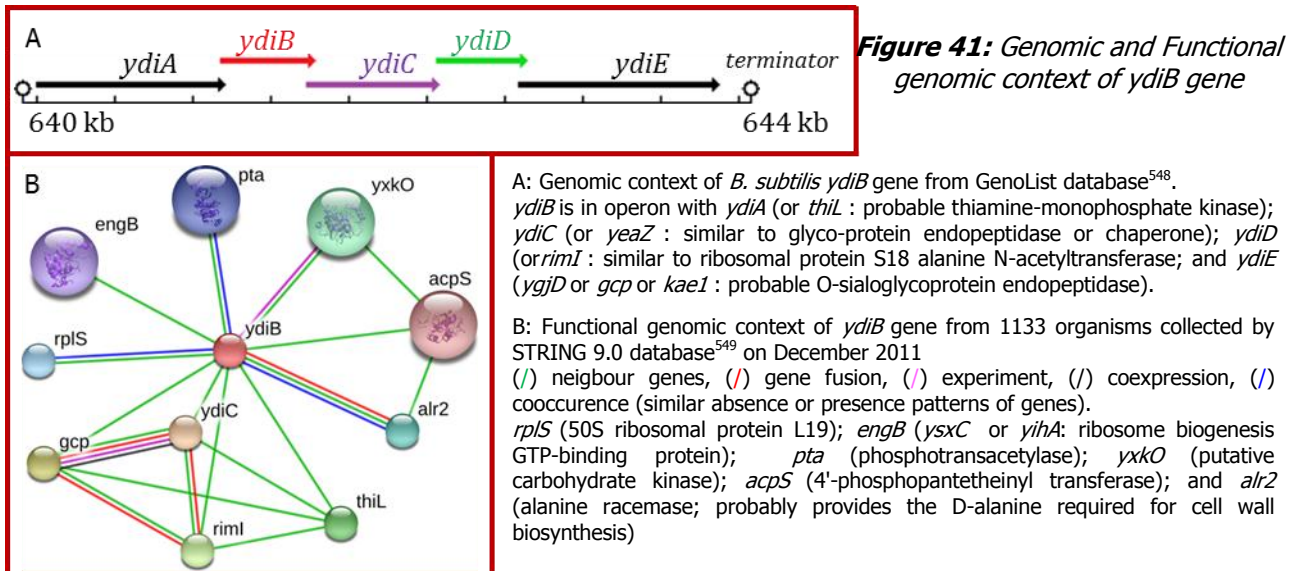
### II.3. Identification of YdiB potential cellular partners

In the section II.2 of this *Results-Discussion* part, we attempted to characterize the enzymatic kinase activity of YdiB at molecular level. From this section henceforward, we will deal with the question: in which *in vivo* biochemical pathway does YdiB take place?

Cellular partner "hunting" is one of the promising strategies to cope with this challenge. Pieces of information obtained from potential partner(s) of YdiB will undoubtedly increase our knowledge about YdiB function. And *if serendipity comes along the way*<sup>546</sup> with a partner of known function, one can expect to reveal the journey of YdiB inside the cell.

#### II.3.1. YdiB and other proteins within its operon

Being very often exposed to an ever-changing environment, bacteria developed an excellent adaptability to survival. Part of this ability lies in operons or clusters of co-regulated genes with related cellular function<sup>547</sup>. Genomic organization thus often suggests some clues about the functions of genes.



According to GenoList database, *B. subtilis* ydiB is the second gene of a five-gene operon ydiABCDE (fig. 41A).

**YdiA** or ThiL is predicted to be a thiamine-monophosphate kinase. However, the gene pair thiL-ydiB is not conserved, since among the total 1059 bacterial and archaeal genomes of OperonDB, these two genes co-occur in the same direction in only 24 genomes<sup>550</sup>.

In the following paragraphs, we will focus on the link between *B. subtilis* YdiB, C, D and E via phosphorylating and dephosphorylating activities.

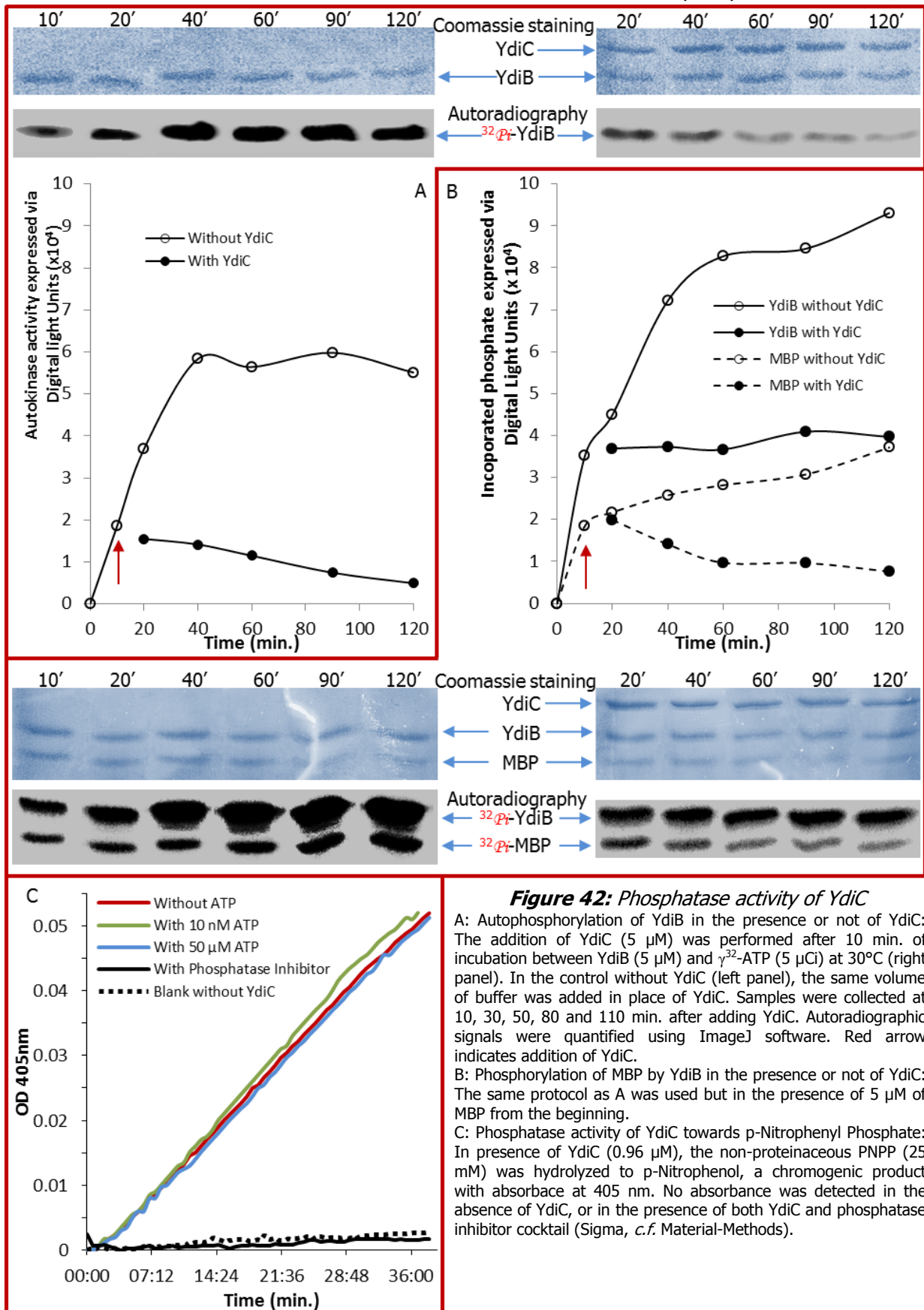
In *B. subtilis*, ydiC is located downstream of ydiB; and ydiD is next to ydiC (fig. 41A), although their counterparts *E. coli* yeaZ (ydiC), yjeE (ydiB) and rimI (ydiD) are not clustered in the same operon. In 2009, Handford & al.<sup>551</sup> in Tracy Palmer's group confirmed the essentiality of yeaZ and yjeE in *E. coli* using inducible promoters allowing programmed depletion of corresponding proteins. YjeE depletion results in increasing the cell size and unusual DNA localization at the periphery while lack of YeaZ leads to enlarged cell coupled with highly condensed nucleoids. Strong interaction between *E. coli* YeaZ and YjeE was also reported via bacterial two-hybrid assay. However, the relationship between *E. coli* YjeE-YeaZ interaction and regulation of their enzymatic activities is still unknown.

We report here the first evidence of enzymatic regulation via interaction between *B. subtilis* YdiB-YdiC. In the presence of YdiC, the signals corresponding to YdiB autophosphorylation were clearly decreased over time (fig. 42A), suggesting a dephosphorylation event. The phosphatase activity of YdiC was then confirmed by PNPP phosphatase activity assay. This PNPP phosphatase activity is indeed specific to YdiC since no absorbance was detected in the absence of YdiC, or in the presence of both YdiC and phosphatase inhibitor cocktail (fig. 42C). Thus, we conclude that YdiC harbours a phosphatase activity and it is plausible that YdiB and YdiC form a functional couple with opposite activities of transferring and removing phosphate groups.

Given that each kinase phosphorylated on average 47 protein substrates in yeast<sup>552</sup>, one can expect that the same situation takes place in bacteria. Assuming that YdiB and/or YdiC in *B. subtilis* would be in a phosphorylation cascade even with the participation of more than one protein substrates, we examined activities of this cognate kinase/phosphatase in the presence of MBP, the *in vitro* substrate of YdiB. The fig. 42B showed that YdiC is capable of dephosphorylating MBP and inhibiting the autokinase activity of YdiB. The interesting fact of this result lies in the function of both YdiB and YdiC towards the same substrate MBP, re-enforcing that these two proteins probably participate in the same biochemical

pathway. Thus, it remains to identify the endogenous substrate of these two enzymes to validate this hypothesis.

...*YdiC* harbours phosphatase activity ...



**Figure 42: Phosphatase activity of *YdiC***

A: Autophosphorylation of YdiB in the presence or not of YdiC: The addition of YdiC (5  $\mu$ M) was performed after 10 min. of incubation between YdiB (5  $\mu$ M) and  $\gamma$ - $^{32}$ -ATP (5  $\mu$ Ci) at 30°C (right panel). In the control without YdiC (left panel), the same volume of buffer was added in place of YdiC. Samples were collected at 10, 30, 50, 80 and 110 min. after adding YdiC. Autoradiographic signals were quantified using ImageJ software. Red arrow indicates addition of YdiC.

B: Phosphorylation of MBP by YdiB in the presence or not of YdiC: The same protocol as A was used but in the presence of 5  $\mu$ M of MBP from the beginning.

C: Phosphatase activity of YdiC towards p-Nitrophenyl Phosphate: In presence of YdiC (0.96  $\mu$ M), the non-proteinaceous PNPP (25 mM) was hydrolyzed to p-Nitrophenol, a chromogenic product with absorbance at 405 nm. No absorbance was detected in the absence of YdiC, or in the presence of both YdiC and phosphatase inhibitor cocktail (Sigma, *c.f.* Material-Methods).

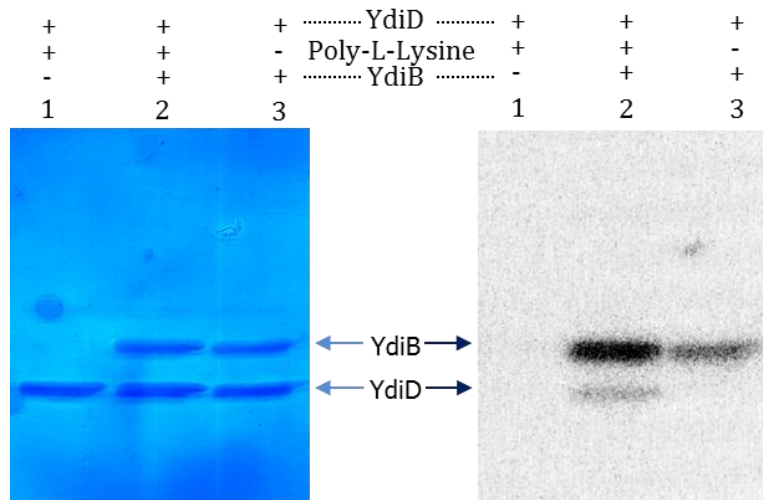
*ydiD* or *rimI* is predicted to acetylate the N-ter alanine of ribosomal protein S18 and in some cases were found to be fused with the gene upstream or downstream (*fig.* 41B, red line *rimI-ydiC* and *rimI-gcp* respectively).

In *E. coli*, knock-out *rimI* mutant exhibits no distinguishable phenotype compared to the wild type, suggesting that this gene is not essential<sup>553</sup>.

RimI belongs to GNAT (*i.e.* Gcn5-related N-acetyltransferase) superfamily, one of the largest enzyme superfamilies recognized to date. The crystal structure availability for more than 24 members revealed the extraordinarily conserved GNAT fold allowing binding of acyl-CoA<sup>554</sup>. Very few of the totals of over 10,000 identified GNAT members in all kingdoms of life have been functionally characterized. Among 26 predicted GNATs in *E. coli* K12<sup>555</sup>, only one has been biochemically characterized as a spermine/spermidine N-acetyl-transferase<sup>556</sup>. Three others, *E. coli* RimI, RimJ, and RimL have been proposed to acetylate the ribosomal proteins S18, S5, and L12, respectively<sup>553,557,558</sup>. The ribosomal protein S18, the putative substrate for RimI, is a very basic protein in the central platform-domain 16S rRNA of the 30S ribosomal subunit<sup>559</sup>. S18 interacts tightly with the ribosomal protein S6 to form a functional heterodimer<sup>560</sup>.

We report here, as shown in *fig. 43*, that YdiB is capable of phosphorylating YdiD in the presence of the activator Poly-L-Lysine, suggesting that YdiD is a potential cellular substrate of YdiB. Given the close link between YdiD (RimI) and ribosome, between polyamine and ribosome (*c.f.* II.2.3 for more details) and the relationship of substrate-kinase between YdiD and YdiB in the presence of poly-L-lysine, we anticipate that YdiB might be implicated in synthesis and/or function of ribosome.

...YdiD is potential cellular substrate of YdiB...



**Figure 43:** Phosphorylation of YdiD by YdiB in presence of poly-L-lysine

YdiD (5  $\mu$ M) was incubated with  $\gamma$ -<sup>32</sup>P-ATP (5  $\mu$ Ci) in the presence or not of YdiB (5  $\mu$ M) and of poly-L-lysine (0.1  $\mu$ M) during 10 minutes at 30°C. After electrophoresis in denaturing condition, the gel was stained by coomassie blue (left panel) before submitted to autoradiography (right panel) using PhosphoImager (PerkinElmer). In the presence of poly-L-lysine, the autokinase activity of YdiB is increased (lane 2 versus lane 3) and YdiD is phosphorylated by YdiB in this condition (lane 2).

While *ydiB* and *ydiC* are well conserved among eubacteria, ***ydiE*** (*ygjD*) orthologs are also found in archaea and eukaryote.

Historically, *ygjD* was first discovered in *E. coli* in 1987 as a monocistronic gene named *orfX* located upstream of *rpsU-dnaG-rpoD* operon encoding ribosomal protein S21, DNA primase, and  $\sigma$ <sup>70</sup> respectively. Later, it was shown to have 76% of identity with a secreted *Pasteurella haemolytica* O-sialoglycoprotease (*i.e.* specific to O-sialic acid-containing glycoproteins)<sup>561</sup>. This activity which leads to its name *gcp*, however might not reflect the ubiquity of *ydiE*, since sialic acids are hardly found in other organisms than vertebrates<sup>562</sup>. Together with *yihA* (so-called *ysxC* or *engB*, *fig. 41B*), *ygjD* were first classified in 1998 as essential genes in both *E. coli* and *B. subtilis* by comparative genomics<sup>563</sup>. With the same approach used with *yjeE* and *yeaZ*, Handford & al.<sup>551</sup> initially reported that *E. coli* without *ygjD* expression displayed similar phenotype as observed in cells lacking YjeE, but in a manner somewhat more heterogenous with both elongated and shortened cells. One year after, in 2010, Katz & al.<sup>562</sup> observed homogenous short *E. coli* cells under YgjD depletion conditions. Recently, using high temporal resolution at the single-cell level, Bergmiller & al.<sup>564</sup> revisited the consequences of depletion of the YgjD protein in *E. coli*. They confirmed a marked decrease in cell size, in parallel with cell division termination as well as stringent response caused by (p)ppGpp induction upon turning off YgjD expression.

In 1999, YdiE family was predicted by Aravind & Koonin<sup>565</sup> to possess the Hsp70 actin-fold, thus might serve as ATP and/or metal-dependent protease with chaperon activity. However, up to date there is no biochemical data support this function.

In 2006, two groups independently reported yeast YgjD homologs as a part of a five-component complex called KEOPS/EKC (*i.e.* **K**inase, **E**ndopeptidase and **O**ther **P**roteins of **s**mall **S**ize/**E**ndopeptidase-like and **K**inase associated to transcribed **C**hromatin) including a kinase and three other small polypeptides<sup>566,567</sup>. YgjD therefore was ascribed the name Kae1 (*i.e.* **K**inase-associated **e**ndopeptidase). While Kisseleva-Romanova & al.<sup>566</sup> reported the involvement of EKC complex in *S. cerevisiae* transcription<sup>566</sup>, Downey & al. found that KEOPS complex promoted both uncapping and elongation of telomeres<sup>567</sup>. In most bacteria, chromosomes are mainly circular and, thus, do not need telomere to protect DNA from degradation. The

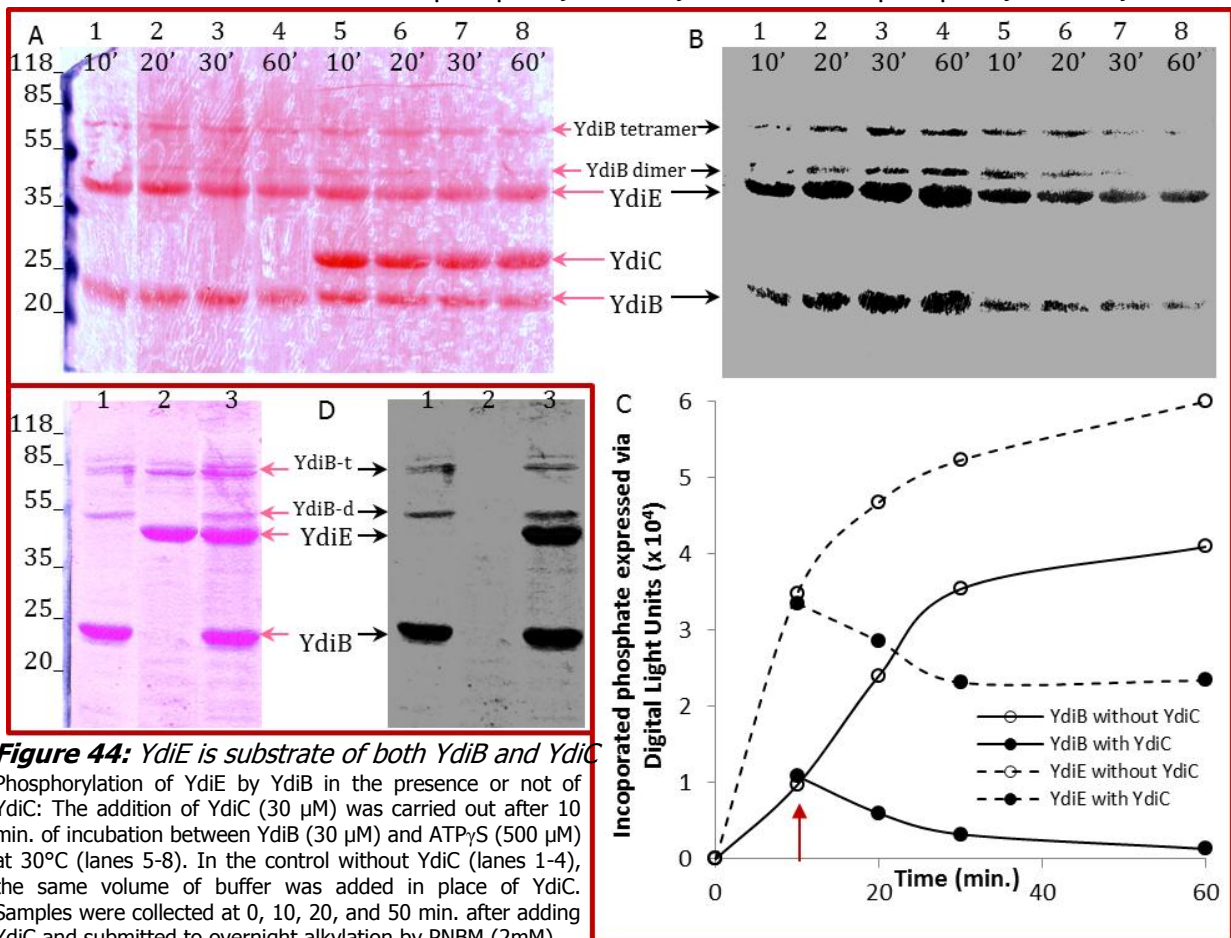


fact that YgjD is also well conserved and essential in prokaryotes suggests other biological function of YgjD in bacteria.

In 2011, Hashimoto<sup>568</sup>, Srinivasan<sup>569</sup>, and Yacoubi<sup>570</sup> separately reported the essential role of both yeast and *E. coli* YgjD in t<sup>6</sup>A modification, the universal tRNA modification N<sup>6</sup>-threonyl-carbamoyl-adenosine consisting of attaching a threonine and a carbonyl group to the amino group of adenine at nucleotide A37, 3' to the anticodon. This t<sup>6</sup>A<sub>37</sub> modification is found in all tRNAs pairing with Axx codons of mRNA in order to strengthen the A-U codon-anticodon interaction on the ribosome. Such modification is required for proper reading frame maintenance<sup>570</sup> and translocation from the A-site (*i.e.* aminoacyl site) to the P-site (*i.e.* peptidyl site), thus assuring the speed and accuracy of translation<sup>571</sup>. Mao & al.<sup>572</sup> reported a weak but significant ATPase activity of archaea *Methanococcus jannaschii* YgjD. Yeast YgjD was also found to possess ATPase activity which is indispensable for t<sup>6</sup>A modification<sup>569</sup>.

In the following *fig. 44*, we report kinase and phosphatase activities of YdiB and YdiC respectively towards YdiE, suggesting the participation of these three proteins in the same physiological pathway.

...YdiE is phosphorylated by YdiB and dephosphorylated by YdiC...



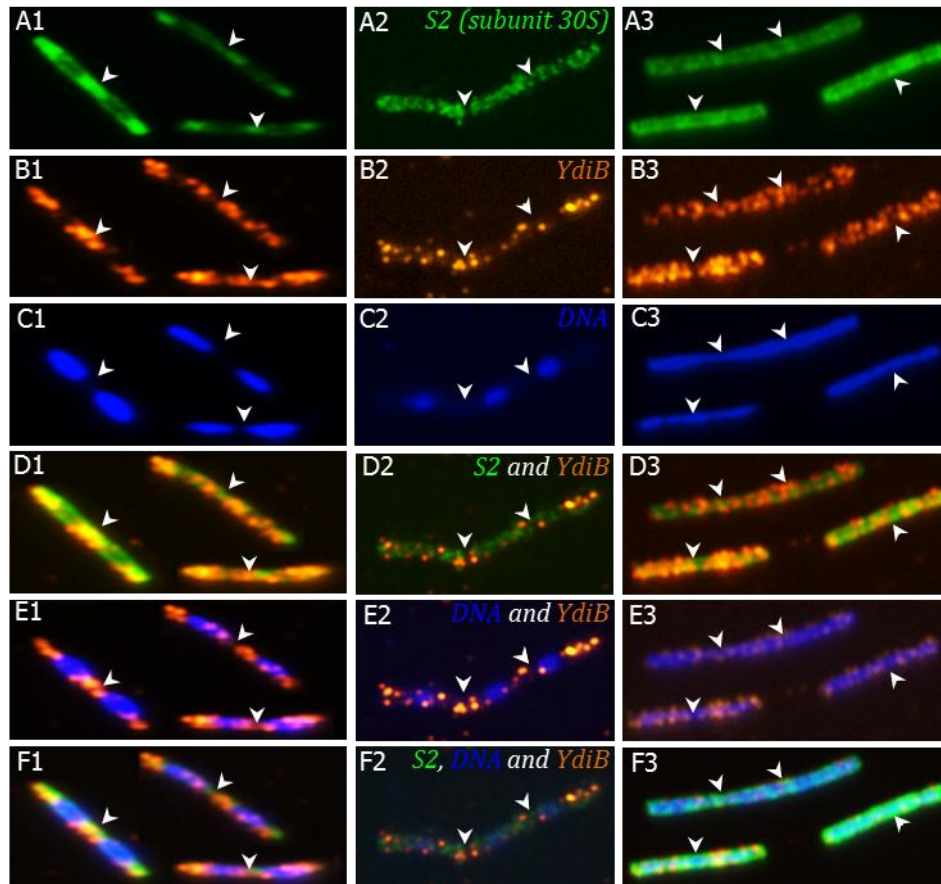
It is worth noting that YeaZ (YdiC) and YgjD (YdiE) share 29% identity within their first 100 amino acids. YdiC also possesses an Hsp70 actin-like fold, but lacks both of ATP- and metal-binding site<sup>573</sup>. In some cases, these two genes were found to be fused together (*fig. 41B*, red line *ydiC-gcp*). It is thus plausible that *ydiC* and *ydiE* are paralogs and during their co-evolution, only one (YdiE) retains the catalytic sites and other (YdiC) provides new surface for additional interaction with other proteins. In agreement with this suggestion, i) recently Yacoubi & al. have shown that<sup>570</sup> YaeZ is required for YgjD function *in vivo* in *E. coli*. These two proteins had been previously reported to be co-purified<sup>574</sup> (*fig. 41B*, black line *ydiC-gcp*), suggesting their strong interaction *in vivo*. ii) We also found that phosphatase activity of YdiC towards PNPP substrate is independent of ATP, suggesting that the lack of ATP-binding site does not affect its phosphatase function (*fig. 42-C*); iii) and in *E. coli*, YdiC was also found to interact with YdiB via both yeast and bacterial two-hybrid<sup>574-576</sup>. However, the latter interaction YdiC-YdiB seems to be strain specific, since it was found neither in *S. aureus* nor in *S. pneumoniae*<sup>576</sup>. Our results in *fig. 42* and *fig. 44* confirmed interactions between YdiB, YdiC and YdiE in *B. subtilis* via phosphorylation and dephosphorylation events. Future works should clarify the relationship between these



events and biological activity of YdiE, *e.g.* i) How phosphorylation of YdiE by YdiB affect  $t^6A_{37}$  formation? ii) If YdiE and/or YdiB and/or YdiC are components of a bacterial complex like KEOPS/EKC?

### II.3.2. YdiB and ribosomal proteins

#### II.3.2.1. Colocalization between YdiB and ribosome



**Figure 45:** Subcellular localization of YdiB, ribosomes and nucleoids in *B. subtilis*

3 growth conditions were compared: without antibiotics (1), with tetracycline (2) and with rifampicin (3)

A: Localization of small-subunit ribosomal protein S2 (encoded by *rpsB*) which was fused to GFP at the C-ter (RpsB-GFP);

B: Localization of endogenous YdiB revealed by antibody anti-YdiB which is recognized by 2<sup>nd</sup> antibody conjugated to Cy3;

C: Localization of nucleoids by DAPI staining; D, E, and F: Overlays of (A & B), (B & C), and (A & B & C) respectively.

White arrows indicate cell limit.

In 1999, Lewis & al.<sup>577</sup> developed a series of plasmid vectors allowing fusions of *gfp* to target gene. One of these vectors was then used to create *B. subtilis* strain mutant 1049 where the gene *rpsB* encoding small-subunit ribosomal protein S2 was fused to *gfp*. Since ~80% of the ribosomes are present as 70S complexes, the green signal tracing S2 localization thus represent actively translating ribosomes<sup>578</sup>. Here we report the use of this strain 1049, a generous gift from Pr. Errington (Sir William Dunn School of Pathology, University of Oxford) to compare the localization of ribosomes and YdiB.

As described earlier<sup>578-580</sup>, ribosomes from untreated cells are concentrated towards the cell poles (*fig.* 45-A1). YdiB detected by immunofluorescence appears to be predominantly located at the poles and also at the periphery of the bacterium (*fig.* 45-B1). This uneven distribution of YdiB was also reported by Karst & al.<sup>10</sup> in our group in 2009 with the wild-type *B. subtilis* strain 168. The localization of YdiB is excluded from the area occupied by the nucleoids (*fig.* 45-C1, E1). Compared to ribosomes, YdiB while is still located at the cell poles, seemingly extends further towards mid-cell (*fig.* 45-B1, D1). In order to see if YdiB strictly follows ribosomes, we treated cells with several inhibitors which have been shown to alter the distribution of nucleoids and ribosomes.

It is admitted that tetracycline, by binding to 30S subunits, prevents the association of aminoacyl-tRNA with ribosomes, thus inhibits protein translation in bacteria<sup>581,582</sup>. In 2006, Hunt & al.<sup>580</sup> reported compact nucleoids and extension of ribosomes from cell poles towards the middle of the cell in response of tetracycline treatment in *B. subtilis* mutant 1049. We observed the same phenomenon with smaller area occupied by nucleoids (*fig.* 45-C2), but different distribution of ribosomes which are heterogeneously dispersed throughout the cell and seemingly still dense at the cell poles (*fig.* 45-A2). The distribution of YdiB is still uneven and its density at the cell poles is more pronounced than ribosomes (*fig.* 45-B2).

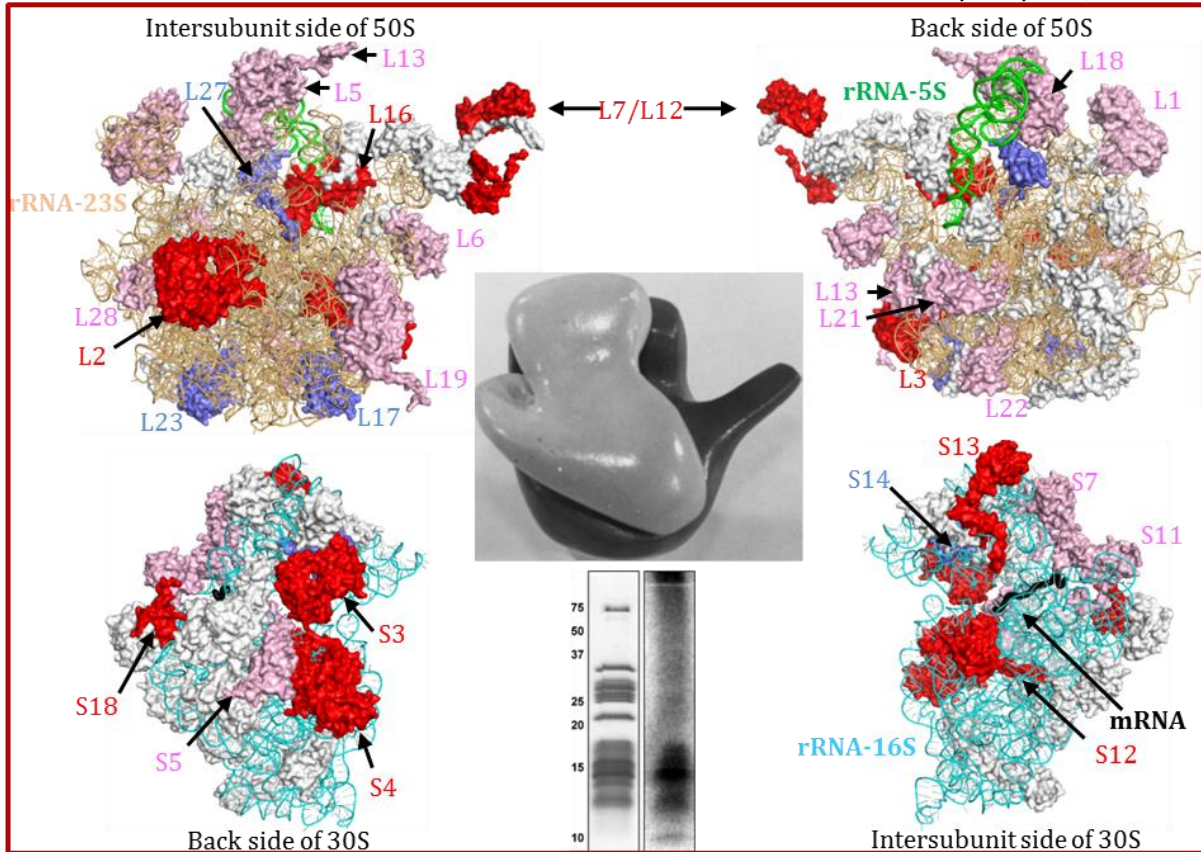
Rifampicin is believed to inhibit DNA-dependent RNA polymerase via binding to its  $\beta$ -subunit, thus preventing RNA synthesis<sup>582</sup>. Treatment of *B. subtilis* mutant 1049 with rifampicin caused inverse effects to tetracycline, where not only DNA (*fig.* 45-C3) but also ribosomes (*fig.* 45-A3) spread out throughout the

entire cell, as reported earlier by Hunt & al.<sup>580</sup>. The distribution of YdiB is also somehow more homologous under this condition (fig. 45-B3). Overlays ribosomes and YdiB revealed peripheral zones where YdiB appears alone without ribosomes (fig. 45-D3).

We thus conclude from the results of these experiments that there is some co-localization between ribosomes and YdiB. This co-localization however, is not *stricto sensu*, since YdiB is also localized in zones without ribosomes. We assume that YdiB might be a protein of multifunction that participates in more than one physiological pathway. One of them might be involved in synthesis or function of ribosomes via phosphorylation control of YdiD and/or YdiE.

### II.3.2.2. Phosphorylation of *B. subtilis* ribosomes by YdiB

...mini review of bacterial ribosome phosphorylation...



**Figure 46:** Structure of bacterial ribosome and phosphorylation of ribosomal proteins

Structures of *Thermus Thermophilus* ribosome 50S and 30S were obtained from the PDB codes 2wrj and 2wri respectively. Ribosomal RNAs were presented as cartoon with different colors (23S in light orange, 5S in green and 16S in cyan). Ribosomal proteins were presented as surface. Phosphorylated ribosomal proteins in *E. coli* and in *Streptomyces* were colored in light pink and pale blue respectively. Red color indicated phosphorylated ribosomal proteins which were found in both *E. coli* and *Streptomyces*. Intersubunit faces of 50S and 30S subunits are presented in top left and bottom right while their back sides (or solvent sides) are found in top right and bottom left, respectively.

The model of 70S ribosome complex in the center is taken from the ref<sup>583</sup>.

The detection of phosphorylated *E. coli* ribosomal proteins from the ref<sup>584</sup> is presented in the bottom center where the left lane corresponds to Coomassie stained gel of ribosomes and the right lane revealed phosphorylated proteins with  $\gamma^{32}$ -ATP.

Three "2009 Nobel Laureates in Chemistry" have produced excellent reviews regarding ribosome structure and function<sup>585-587</sup>. Ribosome, briefly is a protein-producing factory whose 50S peptidyl transferase activity depends not only on 30S binding mRNA, tRNAs, but also on other trigger factors (IF1, IF2, IF3), elongation and translocation factors (EF-Tu, EF-G respectively) as well as release factors (RF1/RF2, RF3). Given that the function of ribosome is strictly related to all these protein factors and also to about fifty constituent proteins (20-21 small subunit proteins and 31-36 large subunit proteins), it is logical to assume that each posttranslational modification (e.g. phosphorylation) on these proteins might regulate the ribosome function.

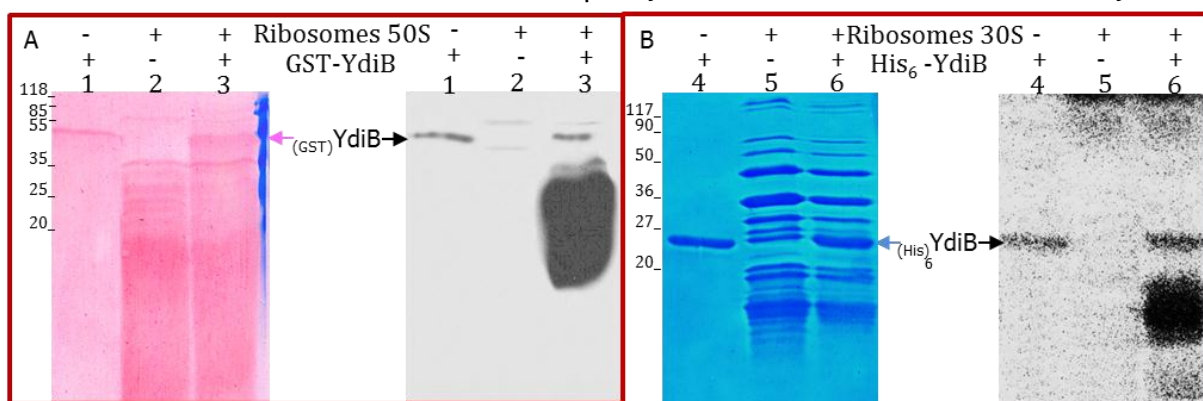
There are pieces of evidence of phosphorylation of *E. coli* IF2<sup>588</sup>, *B. subtilis* and *E. coli* EF-G<sup>28,98,589</sup>, *E. coli* and *Thermus Thermophilus* and *B. subtilis* EF-Tu<sup>28,98,161,590</sup> as well as several 30S and 50S proteins by eukaryotic-like protein kinase in bacteria. Early in 1972, it was reported that *E. coli* ribosomal proteins S4, S9, S18, S19, L2, L3, L5, L7/L12, L10, and L33 were phosphorylated by a protein kinase from rabbit skeletal muscle. In 2009, Soung & al.<sup>584</sup> confirmed and updated this list with phosphorylated S3, S5, S7, S11, S12, S21, L1, L6, L9, L13, L14, L16, L18, L19, L21, L22, L28 and L31 (fig. 46). Phosphosites in target proteins were brought into light with advance of tandem mass spectrometry, but still little is known about the regulation of phosphorylation event towards the biological functions of ribosomes. Finally, protein

kinase(s) which is/are responsible for transferring phosphate groups to Ser, Thr and Tyr sites of these ribosomal proteins is still unknown.

Since 1997, Mikulik & al. have been providing information on phosphorylation of ribosomal proteins in *Streptomyces*. S3, S4, S12, S13, S14, S18, L2, L3, L7/L12, L16, L17, L21, L23 and L27 were phosphorylated by a protein kinase associated with ribosomes in both *Streptomyces collinus*<sup>591,592</sup> and *Streptomyces coelicolor*<sup>593</sup> (fig. 46). The mentioned protein kinase is of 59 kDa and is probed by the antibody raised against protein kinase C, suggesting a Hanks-type protein kinase. Interestingly, they not only found that ribosomal subunits association was influenced, but also that activity to translate poly-U into poly-Phenylalanine was also reduced by these phosphorylations of ribosome. More precisely, binding of N-acetyl-Phe-tRNA to the A site (*i.e.* aminoacyl-tRNA binding site) was not affected upon phosphorylation of ribosomal proteins, but the formation of poly-Phe product was twice slower than in non-phosphorylated ribosome. This result could be explained by either reduction of N-acetyl-Phe-tRNA binding to the P site (*i.e.* peptidyl-tRNA binding site) during translocation<sup>594</sup> or retardation of 3'-deacetylated- Phe-tRNA towards the E-site (*i.e.* exit site)<sup>594</sup>. These results provided first clues of involvement of phosphorylation in protein synthesis process.

Here we report that the protein kinase YdiB is able of phosphorylating *in vitro* purified ribosomes, precisely both 30S and 50S subunits of *B. subtilis* (fig. 47). A majority of ribosomal proteins of less than 25 kDa were phosphorylated by both YdiB tagged with either GST or His<sub>6</sub> at the N-ter in the presence of either ATP $\gamma$ S or  $\gamma$ <sup>32</sup>-ATP. The similar phosphorylation pattern was also found in *E. coli*<sup>584</sup>, as reprinted in the (fig. 45).

...Phosphorylation of *B. subtilis* ribosomes by YdiB...



**Figure 47: Phosphorylation of purified *B. subtilis* ribosomes by YdiB**

A: Ribosomes 50S purified from *B. subtilis* (~30  $\mu$ g) and GST-YdiB (5  $\mu$ g) were incubated alone (lane 2 and 1 respectively) or together (lane 3) in the presence of ATP $\gamma$ S (400  $\mu$ M) at 30°C. Samples were collected after 120 min. and submitted to overnight alkylation by PNBM (2 mM). Denaturing gel electrophoresis was processed with 18% of acrylamide. After western blotting using anti-thio-phospho ester as the 1<sup>st</sup> antibody, blotted membrane was colored with Ponceau Red stain (left panel) and were revealed by Western Exposure Chemiluminescent Detection System (Pierce) (right panel).

B: Ribosomes 30S purified from *B. subtilis* (~20  $\mu$ g) and His<sub>6</sub>-YdiB (3  $\mu$ g) were incubated alone (lane 5 and 4 respectively) or together (lane 6) in the presence of  $\gamma$ <sup>32</sup>-ATP (5  $\mu$ Ci) and unlabeled ATP (500  $\mu$ M) at 30°C during 30 min. After electrophoresis in denaturing condition with 16% of acrylamide, the gel was stained by coomassie (left panel) before to be submitted to autoradiography (right panel) using Cyclon Plus PhosphoImager (PerkinElmer®).

The future work will require bidimensional electrophoresis coupled with mass spectrometry in order to determine phosphorylated ribosomal proteins with their phosphosites. The results obtained from (fig. 47) are the first step pointing out the interaction between YdiB and ribosomes as well as they predict the involvement of YdiB in regulating protein translation via phosphorylation of ribosomal proteins. Below are some clues supporting this hypothesis:

YdiB can phosphorylate YdiD (RimI) which possibly acetylates the ribosomal protein S18. The later protein S18 was found to be phosphorylated in both *Streptomyces collinus*<sup>591</sup> and *Streptomyces coelicolor*<sup>593</sup> as well as in *E. coli*<sup>584</sup> and *Lactococcus lactis*<sup>113</sup>. The interaction between a highly flexible and basic N-ter of S18 with the Shine Dalgarno helix in mRNA is important for the formation of initiation complex during translation<sup>595</sup>. The fact that S18 is a very basic protein and was found to be phosphorylated at Ser, Thr and Tyr residues makes it a good potential candidate substrate for YdiB (*c.f.* II.2.1 and II.2.3).

Previous results obtained by Johanna Karst in our group in collaboration with Jérôme Garin (CEA, Grenoble) using Pull-down technic followed by mass spectrometry revealed several proteins co-purified with YdiB (*c.f.* supplementary data). These proteins, which seemingly strongly interact with YdiB, presumably are its potential cellular partners. Two of the nine potential identified partners are ribosomal proteins L1 and S3. L1 binds directly to the rRNA-23S of 50S subunit and is localized near the binding site of the ribosome for the elongation factor EF-Tu. As for S3, this small subunit ribosomal protein binds to mRNA (fig. 46) and thus position this latter for the decoding process. This result suggests that YdiB could interact with both 50S and 30S subunits. Interestingly, L1 was found to be phosphorylated in *E. coli*<sup>584</sup>, *Lactococcus lactis*<sup>113</sup> and *Streptococcus pneumoniae*<sup>596</sup>, whereas S3 is in phosphoproteom of at least four different bacteria, including *E. coli*<sup>584</sup>, *Streptomyces collinus*<sup>591</sup>, *Streptomyces coelicolor*<sup>593</sup> and *Listeria monocytogenes*<sup>597</sup>. Last but not least, in 2006, Arifuzzaman & al.<sup>598</sup> reported S2, S3, S5, S11, and L14 as

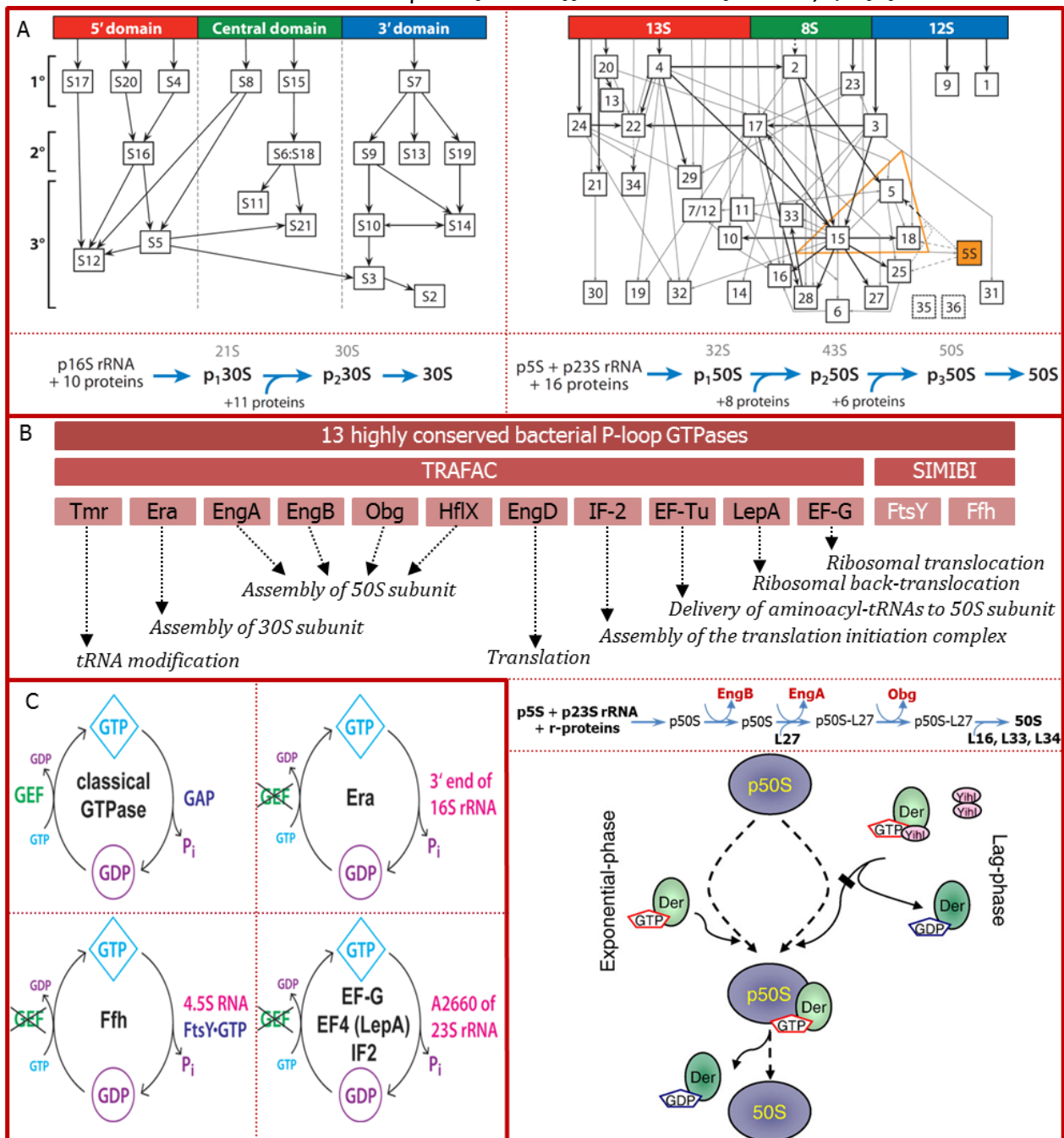


potential partners of YjeE, homolog of YdiB in *E. coli*. Strikingly, all these ribosomal proteins were found to be phosphorylated in *E. coli* by Soung & al. in 2009<sup>584</sup>.

Taken together, these clues lead to the speculation of potential role of YdiB in the regulation of translation via phosphorylation of ribosomal proteins.

### II.3.2.3. Phosphorylation by YdiB of conserved GTPases associated with ribosome

... mini review of ribosome-associated conserved GTPases in bacteria...



**Figure 48:** Involvement of conserved prokaryotic GTPases in ribosome biogenesis and function

A: 30S and 50S assembly maps *in vitro* (upper) and *in vivo* (lower). Pictures were reprinted from Shajani & al.<sup>599</sup>  
 B: General roles of conserved bacterial GTPases in ribosome biogenesis and function (top panel); Preliminary order of action of EngA, EngB and Obg during 50S assembly (middle right panel); Model describing a role of YihI and EngA (here-called Der) during 50S assembly (bottom right panel). This model was adapted from Hwang & Inouye.<sup>600</sup>  
 C: GTPase cycles of classical GTPase and other ribosome-associated GTPases. Pictures were reprinted from Clementi & al.<sup>601</sup>

The newest complete review on universally conserved prokaryotic GTPases was given by Verstraeten & al.<sup>602</sup> in 2011. Based on the classification of Leipe & al.<sup>409</sup> in 2002, 11 of the 13 highly conserved GTPases in total were regrouped in the TRAFAC class (*i.e.* translation factors) and are directly or indirectly implicated in ribosome biogenesis and function, as resumed in the (fig. 48B, top panel).

Ribosome biogenesis is a complex process including five basic steps<sup>599</sup>: (i) transcription, processing, and modification of rRNA; (ii) translation and modification of ribosomal proteins; (iii) proper folding of rRNA and ribosomal proteins; (iv) binding of ribosomal proteins (fig. 48A); and (v) binding and release of assembly factors. GTPases Era (as 30S assembly factor) and conserved GTPases EngA, EngB, Obg and HflX (as 50S assembly



factors) were found to intervene during late stage of ribosome biogenesis, at the fourth and fifth steps. Based on sedimentation rates of ribosomes and ribosomal protein pattern under EngA, EngB and Obg depletion conditions, Karbstein<sup>603</sup> predicted a preliminary order of action with EngB acting before EngA, and Obg as the last GTPase brought into play to achieve a complete 50S subunit. L27 seemingly binds to 50S subunit during EngA functioning and L16, L33 and L34 are gathered thanks to Obg functioning (*fig.* 48B, middle right panel).

Classical GTPases function as molecular switches between an active (GTP-bound) and inactive GDP-bound conformation. Energy released from GTP hydrolysis served for a large variety of biological processes, such as transmembrane signaling<sup>604</sup>, cell division, sporulation, DNA replication, stress response<sup>602</sup> and of course ribosome assembly and function<sup>602,603,605</sup>. However, the intrinsic rate of GTP hydrolysis of classic GTPases is slow, thus additional factors as GAPs (*i.e.* GTPase-activating proteins) are required to rapidly release the energy during GTP hydrolysis. Classical GTPases of Ras type have high affinity for guanine nucleotides and thus need additional factors as GEFs (*i.e.* guanine exchange factors) to substitute the GDP with GTP, bringing the protein in an active state again<sup>601</sup> (*fig.* 48C, top left panel). In term of mechanism of function, ribosome-associated GTPases differ from classical GTPases in two main points:

-Firstly, RNA can serve as a GAP for some ribosome-associated GTPases<sup>601,605</sup>. As shown in (*fig.* 48C), GTPase activities of Era<sup>606-608</sup>, Ffh<sup>609,610</sup>, IF2, EF-G<sup>611</sup> and LepA<sup>612</sup> could be stimulated by ribosomal RNA.

-Secondly, due to weak affinity for guanine nucleotides, the exchange of GDP into GTP occurs with high rate in ribosome-associated GTPases and thus is independent of GEFs<sup>601,603,605</sup>.

In term of structure, GTPases share a common  $\alpha/\beta$  fold of the G domain (*i.e.* GTPase domain) with at least 6  $\beta$  strands forming a central  $\beta$ -sheet surrounded by  $\alpha$ -helices. At the sequence level, G domain is further characterized by the presence of five conserved motifs, G1-G5<sup>602,603</sup>:

-G1 or [Gx<sub>4</sub>GKS/T], so-called the Walker A motif, is shared with other NTPases including some ATPases and kinases and is responsible for binding of  $\alpha$ - and  $\beta$ -phosphates.

-G2, so-called the switch I or effector binding region is highly conserved within each GTPase family but not among different families. Members of TRAFAC class contain a conserved threonine interacting with Mg<sup>2+</sup> that binds to the  $\beta$ - and  $\gamma$ -phosphates.

-G3 or [Dx<sub>2</sub>G], so-called the switch II or the Walker B motif is specific to GTPases among P-loop NTPases. The conserved aspartate and glycine are responsible for Mg<sup>2+</sup> and  $\gamma$ -phosphate binding, respectively. The terms "switch I" and "switch II" refer to the structural difference between GTP- and GDP-bound states observed in Ras-like GTPases. The highly mobile switch regions are often the effector-binding sites.

-G4 or [N/TKxD] determines nucleotide specificity by forming hydrogen bonds with the guanine ring.

-G5 or [SAK/L] is poorly conserved in ribosome-associated GTPases and responsible for recognizing guanine via a hydrogen bond which stabilizes the conserved asparagine and aspartate in the G4 motif.

In addition to the G domain, some ribosome-associated GTPases contain at least one supplementary domain. Despite the diversity in size and sequence of these domains, their common role is involved in ribosome binding via its interaction with either rRNAs or ribosomal proteins. For instance, among 21 GTPases that have been identified so far in *B. subtilis*<sup>602</sup>:

-The essential but not conserved GTPase **YqeH**<sup>613,614</sup> contains a N-ter Zn<sup>2+</sup> binding loop implicated in 16S-rRNA binding<sup>615</sup>.

-The non essential and not conserved GTPase RsgA<sup>616</sup> (also called **EngC**, YjeQ, YloQ) contains a N-ter OB (*i.e.* oligo-saccharide/nucleotide binding) domain interacting with the helices 18, 34, 44 of 16S-rRNA and with the ribosomal proteins S3, S12 of the small subunit. It also contains a C-ter Zn<sup>2+</sup> finger domain interacting with helices 29, 30, 31 of 16S-rRNA as well as with the ribosomal protein S13 of the small subunit<sup>617</sup>.

-The essential and conserved GTPase Obg<sup>614</sup> (also called ObgE, CgtA, YhbZ) contains a conserved N-ter glycine-rich domain favoring protein-protein interaction<sup>618</sup> and a variable C-ter suggested to function as GEF in some biological conditions<sup>619</sup>. The role of these supplementary domains in ribosome assembly remains elusive although there is evidence showing interaction between Obg and 16S and 23S r-RNAs<sup>620</sup> as well as with ribosomal proteins including S3, S4, S5, S9, S13, S16, L2, L4, L13, L16 and L17<sup>620-622</sup>.

-The conserved but not always essential GTPase **Era** (also called Bex, Sgp, or Pra) contains a C-ter KH (*i.e.* nuclear ribonucleoprotein K homology) domain with the consensus motif VIGx<sub>2</sub>Gx<sub>2</sub>I responsible for 16S-rRNA binding<sup>606-608</sup>.

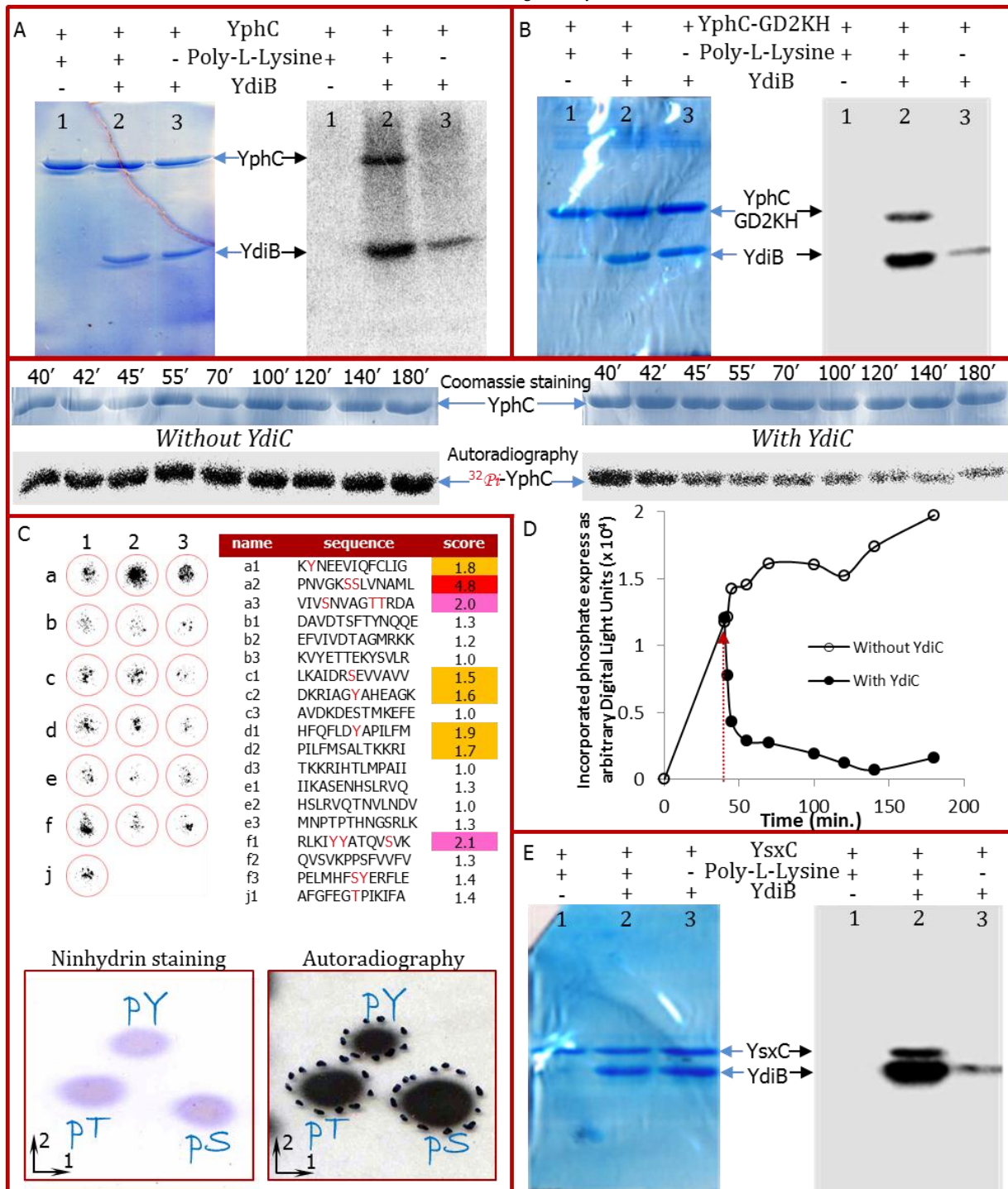
-The essential and conserved GTPase **EngA** (also called Der, YphC, YfgK) is the unique GTPase possessing the two N-ter G-domains in tandem (GD1 and GD2) that do not interact directly with each other but pack at either side of the C-ter KH-like domain<sup>623,624</sup>. This highly basic KH-like domain of EngA lacks the residues responsible for RNA binding and thus might not interact with RNA but instead might bind to ribosome through protein-protein interaction. *E. coli* EngA interacts with 50S subunit via the KH-like domain<sup>625</sup> while *S. typhimurium* EngA interacts with the 30S subunit ribosomal proteins S7 and S9<sup>621</sup>. In 2010, Hwang & Inouye<sup>600</sup> discovered YihI as the first bacterial GAP, which is specific to EngA but not to Obg or Era and capable of recognizing the GTP-bound state of both GD1 and GD2. Based on the result that *yihI* depletion reduces lag phase, it was suggested that YihI associates with GTP-bound EngA during this lag phase on

producing GDP-bound EngA which cannot associate with ribosome. Over time, *yihI* expression is depleted, allowing free GTP-bound EngA to associate with ribosome and fulfill its role (fig. 48B, right bottom).

-The conserved but not always essential GTPase **EngB** (also called YihA, YsxC) contains a single G domain flanked by ~30- and ~60- amino acid stretches at the N-ter and C-ter respectively. In *B. subtilis*, the C-ter 23 residues forms a highly charged region that is essential for EngB function<sup>626</sup> and the "lysine comb" (*i.e.* a row of Lys residues on the solvent-accessible surface) found on this region was suggested to mediate interaction with RNA<sup>627,628</sup>. There is evidence of implication of EngB in the assembly of ribosomal subunits. *S. aureus* EngB co-purifies with ribosomes and interacts with S2, S10, S17 as well as the  $\beta'$  subunit of the RNA polymerase<sup>629</sup>. *B. subtilis* EngB binds L1, L6 and L7/L12 and cells depleted from EngB lack L16, L23 and L36<sup>630</sup>.

...EngA is potential cellular substrate of YdiB and/or YdiC...

...EngB is potential cellular substrate of YdiB...



**Figure 49: Phosphorylation of EngA and EngB by YdiB and dephosphorylation of EngA by YdiC**

A: YphC (EngA, 5  $\mu$ M) was incubated with  $\gamma$ <sup>32</sup>-ATP (5  $\mu$ Ci) in the presence or not of YdiB (5  $\mu$ M) and of poly-L-lysine (0.1  $\mu$ M) during 10 minutes at 30°C. After electrophoresis in denaturing condition, the gel was stained by coomassie (left panel) before submitted to autoradiography (right panel) using PhosphoImager (PerkinElmer).

B: Same as A but YphC was replaced by GD2KH domain of YphC.

C: Phosphosite analysis of GD2KH using biotide assay (top panel) and phosphoamino acid analysis of YphC phosphorylated by YdiB using two-dimensional electrophoresis (bottom panel).

D: Phosphorylation of EngA by YdiB in the presence or not of YdiC: The addition of YdiC (5  $\mu$ M) was realized after 40 min. of incubation between EngA and YdiB (5  $\mu$ M each) with  $\gamma^{32}$ -ATP (5  $\mu$ Ci) at 30°C (right panels). In the control without YdiC (left panels), the same volume of buffer was added in place of YdiC. Samples were collected at 2, 5, 15, 30, 60, 80, 100 and 140 min. after adding YdiC. Autoradiographic signals were quantified using ImageJ software. Red arrow indicates addition of YdiC.

E: Same as A but YphC was replaced by YsxC (EngB).

Here we report that two conserved GTPases implicated in ribosome biogenesis, *B. subtilis* EngA and EngB are phosphorylated *in vitro* by the kinase YdiB in the presence of the activator poly-L-lysine (fig. 49A and E). Furthermore, EngA phosphorylated by YdiB is also a substrate of the phosphatase YdiC (fig. 49D). These results support the implication of YdiB and/or YdiC in ribosome biosynthesis via phosphorylation/dephosphorylation of different components of the biogenesis machinery.

Protein synthesis requires a lot of energy and thus consumes a high proportion of cellular ATP and GTP<sup>631-633</sup>. In mammalian, phosphorylation of ribosome-associated GTPases was proposed to alter the rates of synthesis of specific proteins, particularly at translation initiation and elongation steps<sup>632,633</sup>, in order to quickly respond to changing conditions<sup>632</sup>. In prokaryote, the conserved elongation factor GTPase EF-Tu was first reported to be phosphorylated in both *E.coli* and *Thermus thermophilus* by Lippmanns & al. in 1993<sup>590</sup>. The phosphorylation of EF-Tu was then proposed to abolish its ability to bind aminoacyl-tRNA<sup>329</sup>. In 2009, Absalon & al. in Séror's group found that the Ser/Thr kinase PrkC can phosphorylate EF-Tu *in vitro* at the conserved T384 residue in the presence of basic molecules as MBP or poly-L-lysine. They also demonstrated the *in vitro* dephosphorylation of phosphorylated EF-Tu by PrpC, the cognate phosphatase in the same operon as PrkC in *B. subtilis*<sup>161</sup>. Another conserved translocation factor GTPase, EF-G had been previously proposed to be *in vivo* substrate of *B. subtilis* PrkC and PrpC<sup>163</sup>. Interestingly, *B. subtilis*<sup>634</sup>, *C. crescentus*<sup>635</sup> and *M. tuberculosis* GTPase Obg<sup>636</sup> were found to be autophosphorylated at serine and threonine residues in the presence of GTP but not ATP. Like Obg, *E. coli*<sup>637</sup> and *P. aeruginosa*<sup>638</sup> Era were also found to be autophosphorylated in a GTP- but not ATP-dependent manner at T36, S37 and possibly at S34. The effect of reversible modification by phosphorylation on these conserved ribosome-associated GTPases remains elusive.

The work of A-E. Foucher<sup>639</sup> in our group showed that *B. subtilis* EngA displays a high GTPase activity which originates from the GD1-domain. GD2 is thus proposed to have a different role in the protein's function. As shown in fig. 49B, the purified GD2-KH domain of EngA is phosphorylated by YdiB in the presence of activator poly-L-lysine. Future work should clarify the role of phosphorylation by YdiB on GTPase activity or on other ribosome-related activity of EngA. Given that protein phosphorylation often plays regulator role in protein function, we anticipate that the ribosome-associated function of EngA is regulated via phosphorylation of GD2 domain by YdiB.

There is direct evidence supporting our result of phosphorylation of *B. subtilis* EngA by YdiB. In 2009, *Pneumonas* EngA was shown to be phosphorylated *in vivo* at the residue S34<sup>115</sup>. Another *Streptococcus pneumoniae* phosphoproteomic study in 2010 also brought into light the *in vivo* phosphorylation of EngA at the residue T5<sup>116</sup>. Given that GD2KH is also phosphorylated by YdiB (fig. 49B), we then decided to test the kinase activity of YdiB towards 19 different 13 mer-peptides containing GH2KH-phosphorylatable residues in the middle of their sequence. After incubation with  $\gamma^{32}$ -ATP, the reaction mixture was spotted onto Biotin Capture Membrane which specifically retains biotinylated peptides. After extensive washing to remove unbound components, quantification of signals corresponding to bound and labeled peptides was achieved using PhosphoImager and Gel-Pro Analyser software. Phosphorylation score is expressed as a ratio of each peptide as compared to background peptides. Strong signal corresponding to scores higher than 1.5; 2 and 2.5 were highlighted in orange, magenta and red respectively. As shown in fig. 49C (top panel), GH2KH can be phosphorylated in multiple sites of Ser, Thr and Tyr residues. Among strongest signals are S189, S190 (score 4.8), Y383, Y384, S389 (score 2.1), S204, T209, and T210 (score 2.0). In collaboration with C. Grangeasse (IBCP, Lyon), we confirmed three types of phosphorylatable residues, Ser, Thr and Tyr upon phosphorylation of EngA by YdiB (fig. 49C, bottom panel).

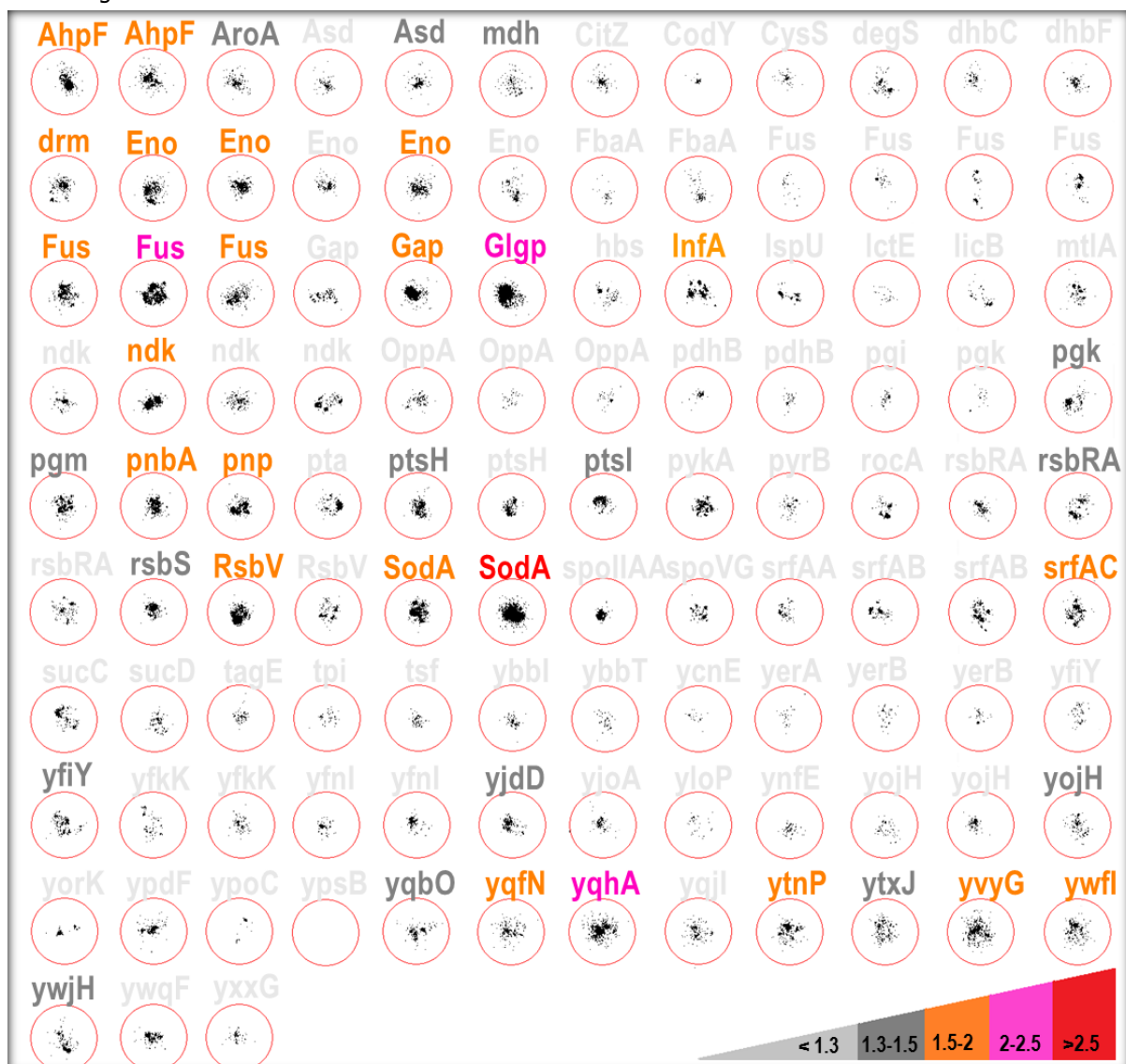
Together with previously presented results, this finding of phosphorylation of the two conserved ribosome-associated GTPase by YdiB strengthens the implication of this new protein kinase in ribosome biogenesis and/or function. From an evolutionary view, this makes sense since the ribosome is an ancient molecular apparatus suggested to be already present in LUCA (*i.e.* the last universal common ancestor of life proposed by Carl Richard Woese in 1998<sup>640</sup>)<sup>601</sup>. Given that gene losses are more common in evolution than gene gains<sup>641</sup>, YdiB, EngA and EngB, as highly conserved proteins interacting with ribosome, thus might also be remnants of evolutionary splits that occurred billions of years ago<sup>642</sup> rather than *de novo* created. Their conservation or possible co-evolution with ribosome therefore is plausibly rationalized as an indispensable requirement for biogenesis and/or function of the protein-making machinery.

II.3.3. YdiB and *B. subtilis* phosphorylatable peptides

Before 2006, different *in vitro* biochemical analyses in *B. subtilis* revealed only 16 phosphosites inside 12 proteins, including SpoIIAA (Ser-58)<sup>643,644</sup>, RsbS (Ser-59)<sup>253,645</sup>, RsbT (Thr-171-205)<sup>646</sup>, RsbV (Ser-56)<sup>647-649</sup>, PrkC (so-called YloP, Thr-162-163-165-167-290-313-320 and Ser-214)<sup>156,158,304</sup>, Idh (so-called CitC, Ser-104)<sup>650</sup>, EF-G<sup>163</sup>, PtkA (so-called YwqD, Tyr-228)<sup>311</sup> as well as its four substrates including two Udgs (YwqF & TuaD)<sup>311</sup> and two Sbs (SsbA, Tyr-82 & SsbB)<sup>312</sup>.

In 2006, Lévine & al.<sup>88</sup> identified a total of 29 *B. subtilis in vivo* phosphoproteins covering 5 of 12 previously mentioned proteins: SpoIIAA, RsbV, EF-G, CitC and PrkC. However they did not identify the location of phosphosites.

In 2007, Macek & al.<sup>28</sup> identified 103 *B. subtilis in vivo* phosphopeptides containing 78 highly confident phosphosites inside 78 phosphoproteins. Four of the 12 previously identified phosphoproteins, RsbT, CitC, PtkA and SsbA were not found in this phosphoproteomic analysis. 78 identified phosphoproteins distribute among a wide variety of metabolic pathways including central carbohydrate metabolism (24%); transport systems and stress response (11% each); protein and nucleic acid biosynthesis (~10% each); antibiotic and cell-wall synthesis (5% each); sporulation (2%). Finally, a large amount of *in vivo* phosphoproteins targeted portions of unknown function (15%), betraying our lack of knowledge on how phosphorylation event regulates cellular function. Linking these new-found phosphorylated proteins to their cognate kinases therefore becomes the big challenge ahead and also the prerequisite step in order to explore biochemical pathways in which these proteins might be involved.



**Figure 50:** *In vitro* phosphorylation by YdiB of peptides containing *in vivo* phosphosites in *B. subtilis*. 111 different 13-mer biotinylated peptides were designed based on 103 *in vivo* phosphopeptides from *B. subtilis* and were then synthesized by BioTides-JPT Peptide Technologies. The peptides were incubated with YdiB in the presence of  $\gamma^{32}$ -ATP before being spotted onto streptavidin-coated Biotin Capture Membrane. Excess radiolabeled ATP was washed away and incorporated  $^{32}$ P signal was measured using PhosphoImager and quantified by Gel-Pro Analyser software. Phosphorylation score is expressed as a ratio of digital light units of  $^{32}$ P incorporated to each peptide to digital light units of background (corresponding to the peptides with the lowest intensity). Signal corresponding to scores superior to 1.3, 1.5; 2 and 2.5 were highlighted in dark grey, orange, magenta and red respectively.



Based on the most comprehensive Ser/Thr/Tyr phosphoproteomic study of *B. subtilis* thus far by Macek & al.<sup>28</sup>, we designed 111 different 13-mer biotides (*i.e.* biotinylated peptides) and tested kinase activity of YdiB towards them. 32 reactions seemingly revealed significant positive signals which were then classified in order of strength as showed in *fig. 50*. The following *tab. 9* aims to analyze and regroup this set of positive biotides in term of biological function of their corresponding proteins. Potential partners are colored in accordance with their corresponding biotides in *fig. 50*.

**Table 9: Potential partners of YdiB revealed by Biotide assay**

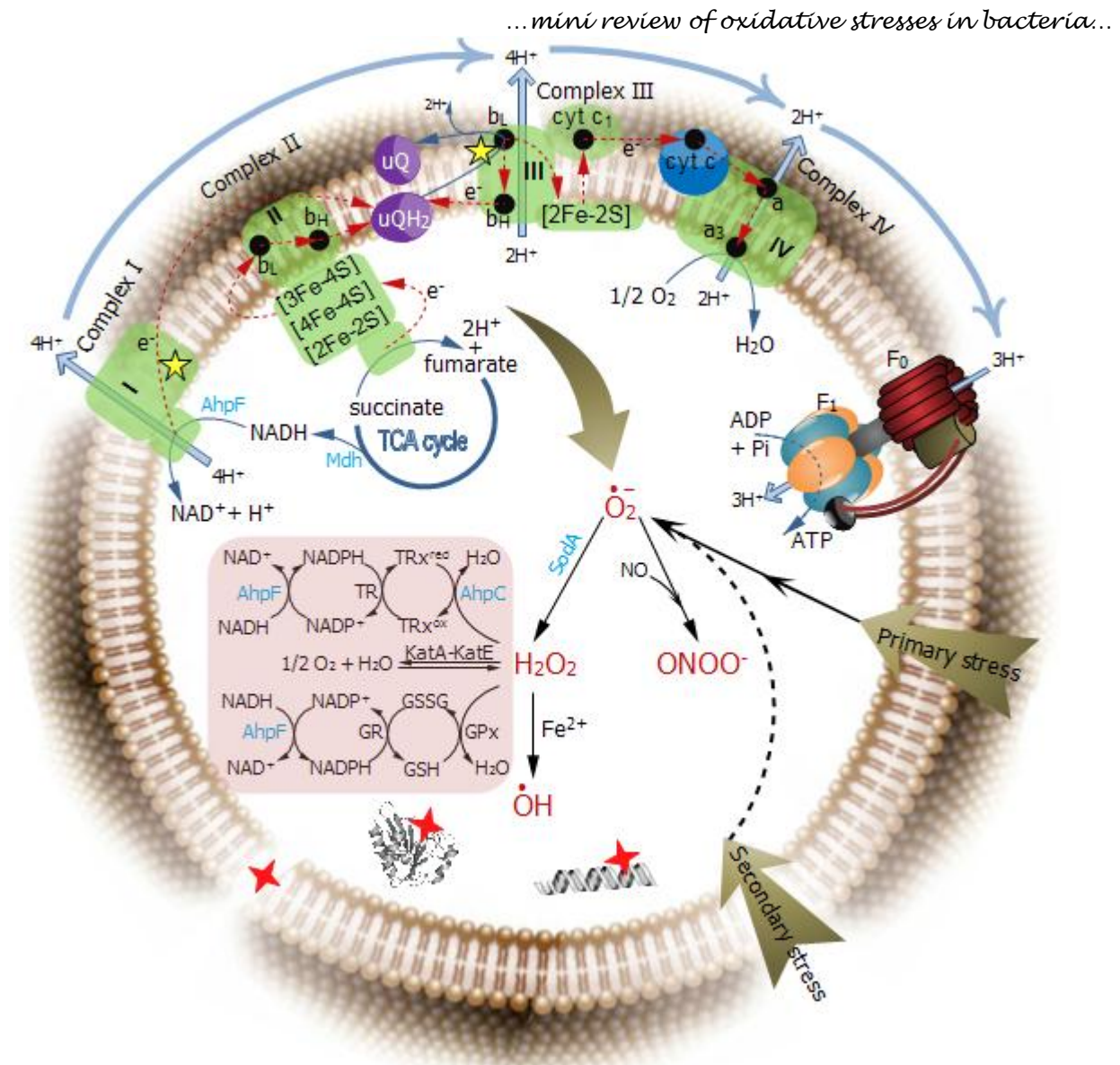
Potential partners	Implication in stress resistance
<b>SodA</b> Superoxide dismutase	-induction upon oxidative stresses (diamide <sup>651</sup> , hydrogen peroxide and paraquat <sup>652</sup> ) -detoxification of oxygen radicals <sup>653</sup> . -co-purification with YdiB by pull-down assay coupled to mass spectrometry <sup>654</sup> - <i>in vivo</i> phosphorylated on Thr-34 and Thr-70 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of SodA on Thr-34 and Thr-70
<b>AhpF</b> large subunit of alkyl hydroperoxide reductase	-induction upon oxidative; heat or salt stresses <sup>655</sup> or limitation of Fe <sup>3+</sup> /Mn <sup>2+</sup> <sup>656</sup> -detoxification of reactive oxygen species (ROS) <sup>657</sup> and electron respiratory chain -co-purification of the cognate small subunit AhpC with YdiB by pull-down - <i>in vivo</i> phosphorylated on Ser-48 or 49 and Tyr-393 or Ser-394 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of AhpF on Ser-48 or 49 and Tyr-393 or Ser-394
<b>HemQ (YwfI)</b> UPF0447, heme-binding protein	-heme biosynthesis and detoxification of peroxide with catalase/peroxidase activity <sup>658</sup> -well conserved in bacteria but absent in eukaryote <sup>658</sup> - <i>in vivo</i> phosphorylated on Ser-35 or -36 or -41 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of HemQ on Ser-35 or -36 or -41
<b>RsbV</b> anti-anti $\sigma$ factor	- <i>in vivo</i> , RsbV is phosphorylated on Ser-52,-56, Thr-57; RsbS on Ser-59; RsbRA on Thr-171 or Ser-181, Thr-205; RsbRC on Ser-165,-174, Thr-186; RsbRD on Thr-181 <sup>28</sup> .
<b>RsbS</b> stressosome protein, antagonist factor	-in unstressed cells, the 1 <sup>st</sup> anti- $\sigma$ factor RsbW phosphorylates RsbV on Ser-56 <sup>647,648</sup> and binds to $\sigma^B$ , preventing it from directing RNA polymerase to $\sigma^B$ -controlled promoters.
<b>RsbRA</b> stressosome protein, co-antagonist $\sigma$ factor	-following energetic stress, the phosphatase RsbP is activated by RsbQ to dephosphorylate <i>p</i> <sub>T</sub> -RsbV <sup>659</sup> , allowing RsbV-RsbW formation and release of $\sigma^B$ for direct transcription of its target genes.
<b>RsbRC (YojH)</b> stressosome protein, co-antagonist $\sigma$ factor	-in unstressed cells, the 2 <sup>nd</sup> anti- $\sigma$ factor RsbT binds to co-antagonist factors and phosphorylates their STAS C-ter domains (RsbRA on Thr-171, RsbRB on Thr-186, RsbRC on Thr-186 and RsbRD on Thr-181 <sup>660</sup> ), forming RsbT-binding stressosome.
<b>RsbRD (YqhA)</b> stressosome protein, co-antagonist $\sigma$ factor	-following environmental stress, RsbT phosphorylates the STAS domain of the antagonist factor RsbS on Ser-59 <sup>660</sup> and disassociates from the stressosome towards the phosphatase RsbU and activates it. RsbU then dephosphorylates <i>p</i> <sub>T</sub> -RsbV and liberates $\sigma^B$ for direct transcription of its target genes. - <i>in vitro</i> , YdiB phosphorylates biotides of RsbV on Ser-52, RsbS on Ser-59, RsbRA on Thr-205, RsbRC on Ser-174 and RsbRD on Thr-181.
Potential partners	Implication in central carbohydrate metabolism (glycolysis, PTS, pentose phosphate shunt, and citric acid cycle)
<b>GlgP</b> Glycogen phosphorylase	-glycogen biosynthesis <sup>661</sup> - <i>in vivo</i> phosphorylated on Thr-291 or Ser-294 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of GlgP on Thr-291 or Ser-294 or Ser-296
<b>GapA</b> Glyceraldehyde -3-phosphate dehydrogenase	-essential glycolytic enzyme that interacts <i>in vitro</i> with Hpr and Crh whose Ser-phosphorylated forms slightly inhibit Gap activity and His-phosphorylated forms could not phosphorylate GapA <sup>662</sup> . -GapA interacts with SR1 (YkzW), a regulatory peptide encoded by small RNA which is expressed under gluconeogenic conditions and repressed under glycolytic conditions <sup>663</sup> - <i>in vivo</i> phosphorylated on Ser-148 or Ser-151 or Thr-153 or Thr-154 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of GapA on Thr-153 or Thr-154
<b>Eno</b> Enolase	-essential, multifunctional and universally conserved enzyme: glycolysis, heat-shock response <sup>664</sup> , RNA degradation <sup>665</sup> , DNA replication <sup>665,666</sup> - <i>in vivo</i> phosphorylated on Thr-141; Ser-259,-325; Tyr-281 or Ser-285 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> phosphorylated by PtkA on Tyr-8, -46, -249, -256, -403, -419, -424, -426 <sup>406</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of Eno on Thr-141, Ser-259, Ser-285
<b>Pgk</b> Phosphoglycerate kinase	-glycolytic/gluconeogenic enzyme - <i>in vivo</i> phosphorylated on Ser-183 and Thr-299 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of Pgk on Thr-299
<b>Pgm</b> Phosphoglycerate mutase	-glycolytic/gluconeogenic enzyme - <i>in vivo</i> phosphorylated on Ser-62 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of Pgm on Ser-62
<b>HPr (PtsH)</b>	-PTS-dependent sugar transport and carbon catabolite repression ( <i>c.f.</i> I.2.2)

Histidine-containing phosphocarrier protein of PTS	- <i>in vivo</i> phosphorylated on His-15 by PstI <sup>667</sup> , Ser-12 and Ser-46 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> phosphorylated on Ser-12 by PrkC <sup>160</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of HPr on Ser-12
<b>PtsI</b> (EI) Phosphoenolpyruvate-protein of PTS	- transiently autophosphorylates on His-189 in the presence of phosphoenolpyruvate PEP then transfers this phosphoryl group to HPr on His-15 ( <i>c.f.</i> I.2.2) - <i>in vivo</i> phosphorylated on Ser-34 or Ser-36 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of PtsI on Ser-34 or Ser-36
<b>ManP</b> (YjdD) mannose –specific of PTS	- sugar catabolism: mannose uptake and phosphorylation, control of ManR activity <sup>668</sup> - <i>in vivo</i> phosphorylated on Ser-365 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of YjdD on Ser-365
<b>YwjH</b> Transaldolase	- pentose-phosphate pathway - <i>in vivo</i> phosphorylated on Ser-39 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> phosphorylated by PrkC on Ser-55, Thr-26, -82, -125, -159, -184 <sup>160</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of YwjH on Ser-39
<b>Mdh</b> Malate dehydrogenase	- TCA cycle and electron respiratory chain - <i>in vivo</i> phosphorylated on Ser-149 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of Mdh on Ser-149
<b>Potential partners</b>	<b>Implication in ribosome function</b>
<b>FusA</b> (EF-G) Elongation factor G	- translation: ribosome translocation - <i>in vivo</i> phosphorylated on Ser-213,-302,-569,-680 and Thr-24 or -25 and Thr-43 or Ser 48 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of FusA on Thr-24 or -25, Ser-302,-680
<b>InfA</b> initiation factor IF-1	- translation: assembly of the translation initiation complex - <i>in vivo</i> phosphorylated on Tyr-60 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> phosphorylated by PtkA on Tyr-60 <sup>406</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of InfA on Tyr-60
<b>TrmK</b> (YqfN) tRNA:m1A22 methyl transferase	- tRNA modification: N <sup>1</sup> -adenosine methylation at position 22 of tRNA <sup>669</sup> - <i>in vivo</i> phosphorylated on Ser-48 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of TrmK on Ser-48
<b>Potential partners</b>	<b>Implication in other biochemical pathways</b>
<b>Drm</b> (YqkN) Phosphopentomutase	- utilization of deoxyribosenucleoside in nucleoside catabolic pathway <sup>670</sup> - <i>in vivo</i> phosphorylated on Thr-87 or Thr-89 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of YvyG on Thr-87 or Thr-89
<b>Ndk</b> Nucleoside diphosphate kinase	- synthesis of nucleoside triphosphates other than ATP - <i>in vivo</i> phosphorylated on Thr-92 and Ser-118 or -120 or -123 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of Ndk on Ser-118
<b>SrfAC</b> (ComL) Surfactin synthase subunit 3	- antibiotic biosynthesis - <i>in vivo</i> phosphorylated on Ser-1003 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of SrfAC on Ser-1003
<b>YtnP</b> lactonase-homolog protein	- defense against competing bacteria <sup>671</sup> - <i>in vivo</i> phosphorylated on Ser-36 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of YtnP on Ser-36
<b>YvyG</b> (YviC) Unknown function	- putative flagellar chaperone <sup>672,673</sup> - <i>in vivo</i> phosphorylated on Tyr-49 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> phosphorylated by PtkA on Tyr-49 <sup>406</sup> - <i>in vitro</i> , YdiB phosphorylates biotides of YvyG on Tyr-49
<b>AroA</b> (AroG) DAHP synthase	- biosynthesis of aromatic amino acids: Phe, Tyr, Trp - <i>in vivo</i> phosphorylated on Ser-2 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of AroA on Ser-2 or Thr-4
<b>Asd</b> Aspartate-semialdehyde dehydrogenase	- biosynthesis of Thr, Lys, dipicolic acid, peptidoglycan <sup>674</sup> - <i>in vivo</i> phosphorylated on Ser-98 and Tyr-146 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of Asd on Tyr-146
<b>YfiY</b> Probable siderophore-binding lipoprotein	- part of the ABC transporter complex yfiYZ/yfhA/yusV involved in import of the iron-hydroxamate siderophores schizokinen, arthrobactin and corprogen <sup>675</sup> - <i>in vivo</i> phosphorylated on Ser-290 and Thr-302 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of YfiY on Thr-302 or Thr-303
<b>YqbO</b> Uncharacterized	- <i>in vivo</i> phosphorylated on Ser-970 and Ser-972 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of YqbO on Ser-970 and/or Ser-972
<b>YtxJ</b> (Csb40) Uncharacterized	- <i>in vivo</i> phosphorylated on Thr-93 or Ser-94 or Ser-96 or Thr-99 in <i>B. subtilis</i> <sup>28</sup> - <i>in vitro</i> , YdiB phosphorylates biotide of YtxJ on Thr-93 or Ser-94 or Ser-96 or Thr-99

In light of these biotide results, a list of corresponding potential substrates of YdiB is now being under construction in order to confirm their *in vitro* phosphorylation by YdiB. We anticipate that YdiB is a protein kinase which may have global importance in *B. subtilis* thanks to its capacity of phosphorylating proteins involved in fundamental cellular processes such as stress resistance, central carbohydrate metabolism, protein synthesis etc... Opening the YdiB's puzzle might therefore leads towards uncovering a big picture where different separate puzzles communicate via phosphorylation and signal transduction.

The last chapter of this manuscript will focus on the involvement of YdiB in oxidative stress resistance via phosphorylation of the superoxide dismutase SodA.

#### II.4. Implication of YdiB in oxidative stress



**Figure 51: Oxidative stress in aerobic bacteria: origin, damage and defense**

ATP, the energy currency of life is synthesized by the ubiquitous F<sub>1</sub>F<sub>0</sub>-ATP synthase machinery<sup>676</sup>, whose driving force resulted from the proton gradient built up during the passage of electrons across the membrane between complexes of the electron transport chain (ETC). In contrast of Photosynthetic ETC which is restricted among plants and some bacteria, all living organisms possess Respiratory ETC composed of ubiquinone pool (uQ) and four complexes (I-IV)<sup>677,678</sup> with different final  $e^-$  acceptors and starter  $e^-$  donors. The main  $e^-$  donor is NADH which feeds  $e^-$  to the complex I via action of enzyme NADH dehydrogenase (AhpF in *B. subtilis*). NADH is generated in the TCA cycle by three enzymes including Malate dehydrogenase (Mdh). During TCA cycle,  $e^-$  derived from oxidation of succinate to fumarate via FAD tunnels along the Fe-S centers and two hemes ( $b_L$ ,  $b_H$ ) of the complex II<sup>679,680</sup>. Leaving the first two complexes,  $e^-$  crosses the ubiquinone pool before entering into the complex III composed of two cytochromes ( $b$ ,  $c_1$ ) with three hemes and a Fe-S cluster. In aerobic bacteria,  $O_2$  is the last  $e^-$  acceptor which obtains  $e^-$  previously transferred from cytochrome c to two hemes of the complex IV. See the text for more details.

$b_L$ , low-potential heme b;  $b_H$ , high-potential heme b; TR, thionin reductase; TR<sup>ox</sup>, oxidized thionin; TR<sup>red</sup>, reduced thionin; GSH, glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; GPx, glutathione peroxidase.



Under normal physiological conditions, O<sub>2</sub> can readily abstract e<sup>-</sup> from one-electron oxidation-reduction reactions to form anion superoxide O<sub>2</sub><sup>-•</sup>, *e.g.*:

- ETC, especially the complexes I and III (indicated by a yellow star ★ in *fig. 51*) are dominant source of cellular O<sub>2</sub><sup>-•</sup><sup>681,682</sup>;
- During non-enzymatic protein glycation process, reducing sugars are attached to primary amine-containing amino acids in proteins. Glycated proteins are stable active sites for catalyzing the formation of free radicals<sup>683-689</sup>, including O<sub>2</sub><sup>-•</sup> in the presence of O<sub>2</sub>;
- Oxidation of [4Fe-4S<sup>2+</sup>] clusters at the active sites of dehydratases (*e.g.* aconitase and fumarase in TCA cycle) via the reaction  $[4\text{Fe-4S}]^{2+} + \text{O}_2 \rightarrow [3\text{Fe-4S}]^+ + \text{Fe}^{2+} + \text{O}_2^{\cdot-}$ <sup>683,690-692</sup>.

O<sub>2</sub><sup>-•</sup> can spontaneously and slowly dismutate to hydrogen peroxide H<sub>2</sub>O<sub>2</sub><sup>693</sup> or is enzymatically dismutated by superoxide dismutases<sup>694</sup> (SodA, SodF and YojM in *B. subtilis*)<sup>652,695</sup>:  $\frac{1}{2} \text{O}_2^{\cdot-} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}_2$ .

H<sub>2</sub>O<sub>2</sub> then is detoxified by different pathways including catalases (KatA and KatE in *B. subtilis*)<sup>696,697</sup>, thio-redoxin system Trx (AhpF/AhpC in *B. subtilis*)<sup>656,698,699</sup> or gluta-redoxin Grx system<sup>700,701</sup>; pink box in *fig. 51*.

In the presence of H<sub>2</sub>O<sub>2</sub>, Fe<sup>2+</sup> released from oxidation of [4Fe-4S<sup>2+</sup>] clusters will trigger hydroxyl radical 'OH production<sup>702,703</sup> via Fenton reaction:  $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{Fe}^{3+} + \text{OH}^-$

ROS (*i.e.* reactive oxygen species: O<sub>2</sub><sup>-•</sup>, H<sub>2</sub>O<sub>2</sub>, 'OH) therefore are products of normal metabolism. In physiological condition, a moderate and controlled production of ROS can lead to reversible oxidation indispensable for redox homeostasis. In this case, ROS acts as beneficial "redox messengers" for signals derived from metabolism and from the environment<sup>175,706</sup>.

However, in case of ROS overproduction, the antioxidant scavengers (SODs, catalases, thioredoxin and glutaredoxin systems) will be overwhelmed, resulting in oxidative stress which provokes non-specific irreversible oxidation of biological molecules, conducting to fatal lesions in cells. Proteins are major targets which traps ~50-70% ROS<sup>176</sup>, but polyunsaturated fatty acids in lipid bilayer membrane are also subject of peroxidation<sup>683</sup> and DNA damage is consequence of 'OH attack<sup>707-709</sup> (indicated by a red four blade propeller ⚓ in *fig. 51*). Accumulation of ROS is related to human pathology *e.g.* hyperglycemic damages<sup>710</sup>, cancer<sup>711</sup>, and neurodegeneration (Alzheimers, Parkinsons<sup>712</sup>, prion diseases<sup>713</sup>).

ROS overproduction was provoked directly by oxidizing agents (H<sub>2</sub>O<sub>2</sub><sup>655,714,715</sup>, ClO<sup>-</sup>, peracetic acid<sup>716</sup>, diamide<sup>651</sup> and paraquat<sup>717-719</sup>, referred to primary oxidative stress) or indirectly under harsh conditions which result in secondary oxidative stress (heat<sup>720</sup>, acid<sup>721</sup>, salt<sup>722</sup>, antibiotics<sup>723-725</sup>, ionizing radiation<sup>726-728</sup> ...)<sup>693</sup>.

Exposition of cells to H<sub>2</sub>O<sub>2</sub> leads to induction of different enzymes<sup>715</sup>, especially catalase/peroxidase, and alkyl hydroperoxide reductase (AhpC/F)<sup>656</sup>. NAD-dependent malate dehydrogenase (Mdh) was found to protect *E. coli* against oxidative damage caused by either H<sub>2</sub>O<sub>2</sub> or γ-irradiation<sup>729</sup>.

Adding paraquat to *E. coli* cultures results in increase of manganese-superoxide dismutase (MnSOD) biosynthesis<sup>730</sup>, but not iron-superoxide dismutase (FeSOD). Induction of MnSOD but not FeSOD<sup>731</sup> correlates with a gain of resistance towards oxidative stress in *E. coli*. Deletion of *sodA* encoding MnSOD in *B. subtilis* makes both vegetative cells and spores more sensible to paraquat<sup>653</sup>.

One needs to stress out that Mdh<sup>28,98</sup>, AhpC<sup>88,90,98</sup>, AhpF<sup>28</sup>, SodA<sup>28,732</sup> were found to be phosphorylated *in vivo* not only in *B. subtilis* but also in other bacteria. It raises the question whether and how their activities are regulated by phosphorylation event *in vivo*. Given that the four genes encoding these proteins are well conserved in bacteria, the discovery of the novel kinase exclusively conserved in bacteria, YdiB, reveals the putative role of phosphorylation event imposed onto these proteins. Interestingly, biotides of these four extremely conserved enzymes were phosphorylated *in vitro* by YdiB (*c.f. tab. 9*). Furthermore, preliminary results using GST-tag-pull-down approach obtained by J. Karst<sup>654</sup> in our group showed that AhpC and SodA co-purified with YdiB (*c.f. tab. S1-supplementary data*). Given the roles of these proteins in scavenging ROS during oxidative stress, we focus our interest in answering the question of possible implication of YdiB in oxidative stress response.



#### II.4.1. Activity of SodA is regulated upon phosphorylation by YdiB

Since the discovery of superoxide dismutase (SOD) by McCord and Fridovich in 1969<sup>694</sup>, we have gained a very impressive increase in our knowledge of ROS and oxidative stress. Despite the ubiquity of SODs in all living organism, the pattern of SODs differs from eukaryote to prokaryote and from bacteria to bacteria. SODs have been regrouped into four main classes based on their metal specificity: Cu/Zn, Ni, Mn or Fe<sup>733</sup> with three distinguishable folds. MnSODs and FeSODs bears two-domain organization mainly composed of  $\alpha$  helices<sup>734</sup> in contrast to  $\beta$ -barrel in Cu/ZnSODs<sup>735</sup> and quaternary structure organization in NiSODs<sup>734</sup> and Fe/ZnSODs<sup>736</sup>, indicating their independent evolution. Cu/ZnSODs (encoded by *sodC*) are the most abundant SODs in nature, found in all eukaryotes and many prokaryotes<sup>735</sup>. FeSODs (*sodB*) present in bacteria and some plants while Fe/ZnSODs (*sodF*)<sup>737</sup> and NiSODs (*sodN*)<sup>734</sup> are restricted inside *Streptomyces* and *cyanobacteria*. MnSODs (*sodA*) localize in the cytoplasm in bacteria as well as in the cytosol of eukaryotic mitochondria and chloroplasts<sup>683,735,738</sup>. Being ROS scavengers, MnSODs in mitochondria function as tumor suppressors<sup>739,740</sup> while prokaryotic SODs contribute to virulence in many pathogens<sup>741</sup>.

The pathogenous anthrax-causing *B. anthracis* contains four putative SODs<sup>742,743</sup> which were re-assigned by Tu & al. as SodA, SodB, SodC and Sod15 (Fe/Mn specificity)<sup>695,744</sup>. They all contribute to virulence in *B. anthracis*<sup>741,742</sup> with resident endospore SodA and Sod15 and nonresident SodB and SodC<sup>742</sup>. *B. subtilis* is a non-pathogenic stress resistant endospore-forming bacterium that shares a broad similarity in metabolic genes with *B. anthracis*, especially in the sporulation machinery<sup>743</sup>. Beside the two putative SODs (SodF, YojM) which have still been poorly characterized but are similar to FeSOD and Cu/ZnSOD<sup>97,652</sup>, *B. subtilis* contains SodA as the main intracellular MnSOD which is essential for resistance of both vegetative cells and spores to oxidative stress<sup>653</sup>.

In order to response efficiently and rapidly to the fluctuation of  $O_2^{\cdot-}$  concentration, the expression of SODs need to be tightly controlled at not only the (post)-transcriptional but also (post)-translational levels, respectively ensuring a good quantity and quality of SODs. Expression of eukaryotic SODs has been more widely studied<sup>745</sup> than their bacterial counterparts, but still there is information available in bacteria:

In *E. coli*, *sodA* is induced by  $O_2^{\cdot-}$ <sup>746</sup> and abundantly upon paraquat exposure<sup>201,749</sup>. The transcription of *E. coli* SodA is governed by several regulatory factors<sup>750,751</sup> including ArcA, Fnr, Fur, SoxRS. Thus far only the Fur locus was proposed to regulate the transcription of *sodB*<sup>752,753</sup>. In *E. coli* and other bacteria, *sodC* is repressed in exponential phase growth and only expressed during aerobic stationary phase<sup>754-756</sup>.

Based on subcellular localization of different SODs in bacteria, it was proposed that the cytoplasmic SodA and SodB protect cells from endogenous  $O_2^{\cdot-}$  while the periplasmic SodC is principal factor protecting cells against exogenous  $O_2^{\cdot-}$ <sup>755,757</sup>. However, this suggestion needs to be revised given that in *B. subtilis*, SodF and YojM (probably SodC) were not induced after paraquat treatment, in contrast to the remarkable induction of SodA<sup>652,719</sup>. The growth of the *B. subtilis* lacking *sodA* decreases as compared to the wild type in the absence of exogenous oxidative stress and exposure of this mutant to paraquat further impaired its growth, indicating the role of SodA in response to both internal and external sources of  $O_2^{\cdot-}$ <sup>695</sup>.

In *Streptomyces* sp., *sodF* repression in presence of  $Ni^{2+}$ <sup>737</sup> was controlled by two regulatory systems. The down-stream complex SrnQ/R<sup>758</sup> contains SrnQ which senses  $Ni^{2+}$  and active the binding of SrnR to the *cis*-regulatory element (*cre*) up-stream of *sodF*<sup>759</sup>. Another system called Nur (homologue of Fur) can bind to the promoter region of *sodF* in the presence of  $Ni^{2+}$  and thus function as a repressor factor<sup>760</sup>. In contrast of *sodF*, *Streptomyces* sp. *sodN* is upregulated in the presence of  $Ni^{2+}$ <sup>761</sup>, but Nur is seemingly not the direct regulator factor for *sodN*<sup>762</sup>.

One exemplar of post-transcriptional control of SODs is the case of the mitochondrial RNA encoding SodA. The transcription of this RNA is closely mediated by its *cre* in the 3' untranslated region which is recognized by a redox-sensitive cytosolic protein<sup>763</sup>. The binding capacity and therefore the enhancer activity<sup>764</sup> of this latter are interestingly up-regulated via tyrosine dephosphorylation<sup>765</sup>.

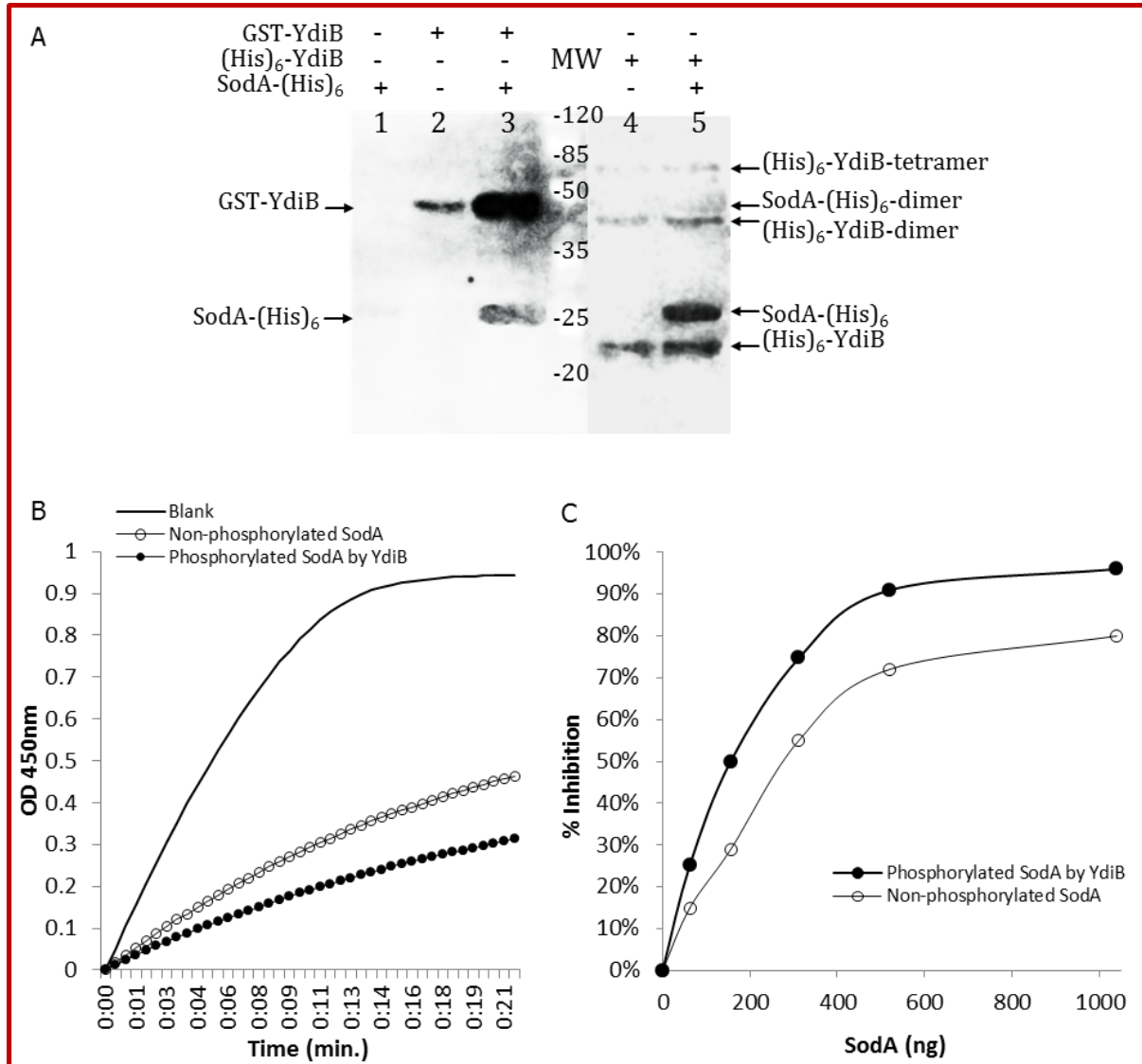
Understanding of post-translational regulation of bacterial SODs has been still extremely limited. The first example was obtained with *Streptomyces* SodN whose NiSOD activity is expressed only upon proteolysis of its N-ter 14 first amino acids<sup>761</sup> by the downstream co-expressed peptidase SodX<sup>766</sup>. The same event was then confirmed in *Prochlorococcus marinus*<sup>767</sup>, suggesting a common activation mechanism in NiSODs. The second example and also the last example thus far was the case of *Listeria* SodA whose MnSOD activity is down-regulated via phosphorylation<sup>768</sup>. *Listeria* SodA was shown to be phosphorylated by bovine heart PKA *in vitro* and dephosphorylated by *Listeria* Stp phosphatase *in vivo* and *in vitro*, however Archambaud & al. were not able to show the *Listeria* kinase responsible for phosphorylating SodA<sup>768</sup>.

Here we report that YdiB is capable of phosphorylating SodA *in vitro* (fig. 52, A), and this phosphorylation event enhances MnSOD activity of SodA (fig.52, B, C). In contrast to *Listeria* SodA, phosphorylation of *B. subtilis* SodA by bovine heart PKA also increases its MnSOD activity was also increased (fig. S2-supplementary data). This observation suggests different mechanism of SOD activity regulation in *B. subtilis* SodA and its counterpart in *Listeria*. Support of this idea, *L. monocytogenes* SodA was recognized by both anti-phosphoSer and anti-phosphoThr<sup>768</sup>, but the release of *L. monocytogenes* phosphoproteome revealed only Ser-63 as phosphosite *in*

*in vivo* for *Listeria* SodA<sup>732</sup>. In contrast, only Thr-containing phosphopeptides of SodA were found in the phosphoproteome of *B. subtilis*<sup>28</sup>, pointing out two phosphosites Thr-34 and Thr-37.

Taken together with the copurification between SodA and YdiB (tab. S1-supplementary data) and the phosphorylation of Thr34 and Thr37-containing biotides of SodA by YdiB, this result underlines SodA as potential *in vivo* substrate of YdiB, thus providing first clue for the possible implication of YdiB in oxidative stress regulation.

...regulation of MnSOD activity of SodA via phosphorylation by YdiB...

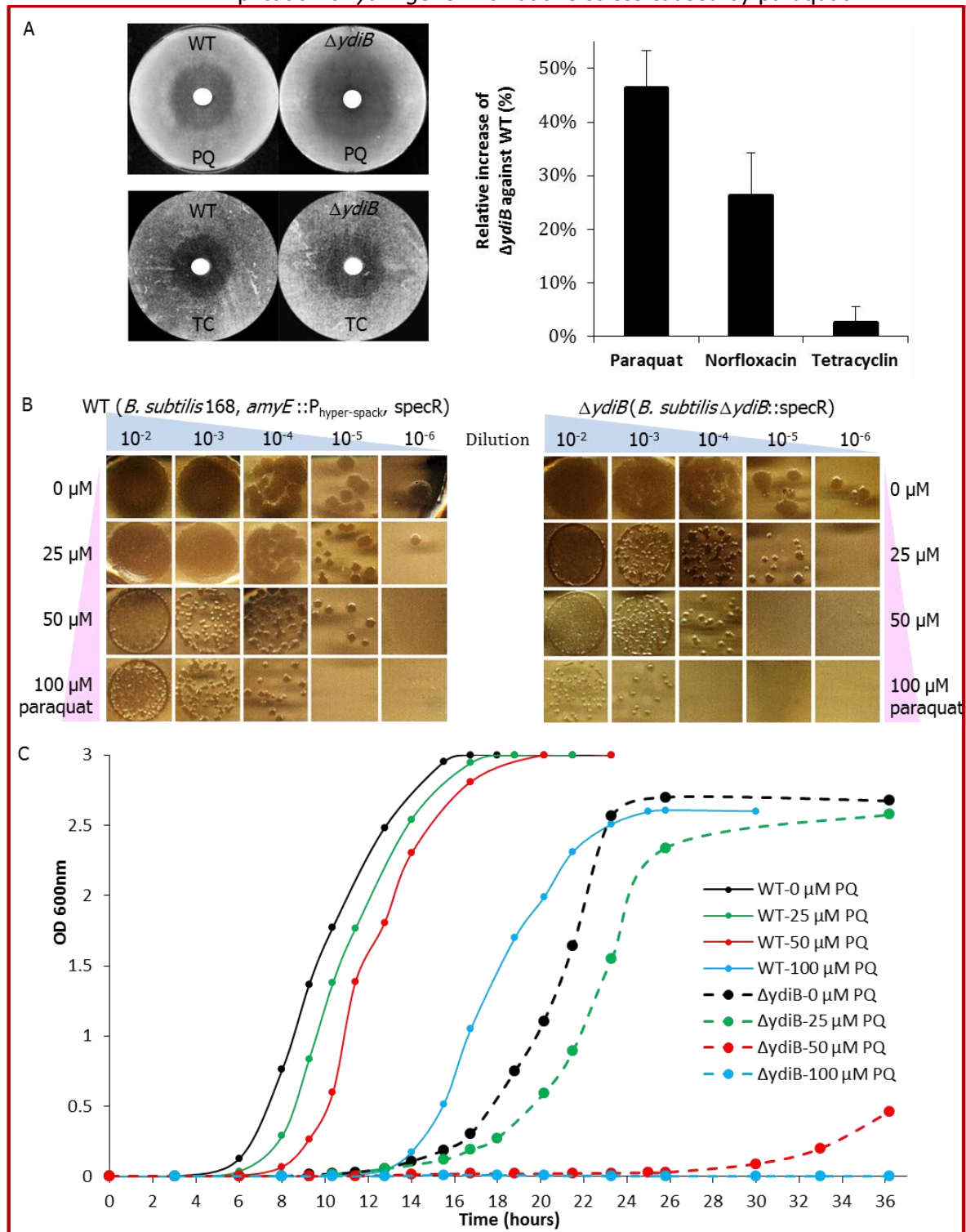


**Figure 52: Superoxide dismutase activity of SodA is increased upon phosphorylation by YdiB**

A: *In vitro* phosphorylation of SodA by YdiB: Purified SodA-(His)<sub>6</sub>, GST-YdiB and (YdiB-(His)<sub>6</sub>) were incubated alone (lane 1, 2 and 4 respectively) or together (SodA + GST-YdiB, lane 3 and SodA + YdiB-(His)<sub>6</sub>, lane 5) in the presence of ATP<sub>γ</sub>S at 30°C overnight, followed by alkylation with 2 mM of PNBM. Western blotting was carried out using anti-thio-phospho ester as the 1<sup>st</sup> antibody, and autographic signals were revealed by Western Exposure Chemiluminescent Detection System (Pierce®).

B: Phosphorylation of SodA by YdiB increases SOD activity: Xanthine Oxidase (XOD) generates superoxide radical resulting in the reduction of WST-1 by superoxide anion to a colored WST-1 formazan product that absorbs light at 450 nm. SOD scavenges superoxide anion thereby antagonizes XOD, reducing the rate of WST-1 formazan generation. SodA (65 µg, 25 µM final) was incubated with ATP (500 µM) in the presence or absence of YdiB (30 µg, 13 µM final) at 30°C overnight. 80ng of SodA (phosphorylated --●-- or non-phosphorylated --○--) was injected to SOD assay system containing WST-1 and XOD. Dilution buffer (supplied in the kit) was used in place of SodA for the blank (—). Formation of WST-1 formazan was followed by absorbance at 450 nm. SOD activity colorimetric assay kit was supplied by Fluka-Simga-Aldrich®.

C: Inhibition curve: The same protocol as B was used with different concentrations of SodA. IC<sub>50</sub> of non-phosphorylated (—○—) and phosphorylated (—●—) forms are 156 and 312 respectively.

II.4.2. Implication of *ydiB* gene in oxidative stress caused by paraquat

**Figure 53: Involvement of YdiB in oxidative stress in *B. subtilis* caused by paraquat**

**A:** Disc inhibition assay. *B. subtilis* strains were grown at 37°C overnight in LB medium, diluted 1:100 into fresh LB medium, and grown at 37°C with shaking. Mid-log phase WT and  $\Delta ydiB$  cultures were harvested and the pellets were then re-suspended into fresh LB medium with 50  $\mu$ g/mL of spectinomycin. 1 mL of fresh WT and  $\Delta ydiB$  cultures (OD<sub>600nm</sub> = 0.14 and 0.148 respectively) were mixed with 50 mL of agar LB (at ~ 40°C) and poured evenly onto a sterile petri dish. After cooling, filter paper disks (7-mm diameter) carrying chemicals to be tested (100 ng of paraquat-PQ, norfloxacin or tetracycline -TC) were placed onto the top of the agar. The diameters of the inhibition zone were measured after the plates were incubated at 37°C for 24 hours.

**B:** Gradient plate assay. *B. subtilis* strains were grown in LB medium at 37°C overnight, diluted 1:100 into fresh LB medium, and grown at 30°C with shaking. Dilutions ( $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$ ) of mid-log phase WT and  $\Delta ydiB$  cultures (OD<sub>600nm</sub> = 0.43 and 0.48 respectively) were spotted onto LB agar plates containing different concentration of paraquat (0-100  $\mu$ M) and incubated at 37°C overnight. This experiment was repeated two times and similar results were observed.

**C:** Growth analysis. One clone of each *B. subtilis* strain (WT and  $\Delta ydiB$ ) was individually re-suspended into 1 mL of LB containing 50  $\mu$ g/mL spectinomycin. 100  $\mu$ L of these bacterial cultures were individually added into 50 mL of LB media containing different paraquat concentration (0-100  $\mu$ M), and grown at 30°C with shaking. Growth curves of WT and  $\Delta ydiB$  are presented in solid and dotted lines respectively.

As previously analyzed, many clues (Mdh, AhpC/F and especially SodA) lead to the hypothesis that YdiB might play a role in oxidative stress response. We then investigate the sensibility of *B. subtilis* wild-type (WT) and the knock-out mutant ( $\Delta ydiB$ ) towards different oxidative stress causing-compounds. Paraquat is well-known to increase the  $O_2^{\cdot-}$  production leading to overproduction of SodA in many bacteria, including *B. subtilis*<sup>652,719</sup>. In 2007, Kohanski & al.<sup>724</sup> reported that bactericidal antibiotic (e.g. norfloxacin) simulates the production of  $OH^{\cdot}$  as a consequence of an oxidative damage pathway. They also showed that this effect is specific to bactericidal antibiotic but not observed in bacteriostatic antibiotic (e.g. tetracycline).

As shown in the (fig. 53-A), no difference was detected between the two strains in regard to tetracycline, but  $\Delta ydiB$  becomes more sensitive to norfloxacin and especially to paraquat. This result agrees with the  $O_2^{\cdot-}$  scavenger activity of SodA to produce  $H_2O_2$ . Since this activity is increased via phosphorylation by YdiB, the deletion of scavenger's activator therefore leads to accumulation of toxic  $O_2^{\cdot-}$ .

Under unstressed anaerobic conditions when  $O_2^{\cdot-}$  could not be<sup>769</sup> or hardly produced, the gene *ydiB* is dispensable and thus no difference was observed between the growth of WT and  $\Delta ydiB$  (not shown). This was also the case observed in *YjeE*, the homologue of YdiB in *E. coli*<sup>770</sup>.

Under unstressed aerobic conditions, concentration of  $O_2^{\cdot-}$  increases and thus YdiB is probably required for optimizing SodA activity. Deletion of *ydiB* therefore displays an effect in slow-downing the bacterial growth (fig. 53-C, native condition of 0  $\mu$ M paraquat). However, this effect of *ydiB* deletion might be alternatively explained by other pathways in which YdiB might be involved in, such as in assuring the ribosome's function. Thus other experiment, such as exposure of WT and  $\Delta ydiB$  to oxidative stress condition which can increase dramatically  $O_2^{\cdot-}$  concentration will help to clarify this ambiguity.

Upon exposure of *B. subtilis* to paraquat, the increase of  $O_2^{\cdot-}$  concentration up to a toxic level triggers the response system including overexpression of SodA and up-regulation of its activity, possibly via phosphorylation by YdiB, which more than ever becomes indispensable. The absence of YdiB therefore makes the mutant much more sensitive to paraquat, as confirmed by following the growth of WT and  $\Delta ydiB$  in a paraquat dose-response manner in both solid and liquid media (fig. 53-B and C).

The lack of difference of  $\Delta ydiB$  in comparison with WT towards tetracycline or under anaerobic condition (no over-production of  $O_2^{\cdot-}$ ), as well as the sensitivity of  $\Delta ydiB$  in comparison with WT towards paraquat and norfloxacin (induction of  $O_2^{\cdot-}$  and thus  $OH^{\cdot}$  as the final lethally toxic product) confirmed that the protective role of YdiB is linked to an  $O_2^{\cdot-}$  scavenger activity. Supporting this conclusion is the results of norfloxacin and other fluoroquinolones induced-expression of *E. coli yjeE*, obtained in 2008 by Mangat & al.<sup>770</sup>; along with up-regulation of *E. coli sodA* following norfloxacin or natural peptide toxin CcdB treatment, as previously shown by Dwyer & al.<sup>771</sup>. All of these compounds are in fact derivatives of fluoroquinones which inhibit DNA gyrase and induce oxidative damage via production of  $O_2^{\cdot-}$  and  $OH^{\cdot}$ . Involvement of YdiB in response to over-generation of  $O_2^{\cdot-}$  therefore seemingly is not restricted in *B. subtilis* but might refer to a common mechanism of bacterial defense to superoxide stress.

Our results thus open a new chapter in which the novel uncharacterized but well-conserved protein kinase YdiB expresses its essential role in protecting cells against oxidative stress caused by  $O_2^{\cdot-}$  overproduction. We suggest that in *B. subtilis*, this role is directly implicated in the capacity of up-regulating superoxide dismutase activity of SodA via phosphorylation. Given the special pattern of the gene distribution of *ydiB*, each puzzle opened from the *ydiB* niche could lead, not only to a better understanding of fundamental biology, but also to open the door for a new generation of antibiotics.



## ...working hypothesis on the cellular function(s) of YdiB...

In light of the results obtained during this study, we suggest that the conserved protein kinase YdiB might play a role in regulating ribosome biosynthesis and/or function under normal physiological condition; and in protecting cells from oxidative stress. In the models presented in the (fig. 54), we propose that:

Under unstressed condition, EngA, EngB, YdiD and YdiE are four potential substrates of YdiB that possess different ribosomal-related activities:

The conserved GTPase EngA containing two G-domains in tandem interacts with ribosomal proteins (possibly in both 50S and 30S subunits). The GTPase activity originates from the GD1-domain, whereas the GD2-KH domain is proposed to have a regulatory role in the protein's function possibly controlled via phosphorylation by YdiB at different Ser/Thr/Tyr residues. In support of this contention, EngA presents in form of phosphor-Thr-protein in *Pseudomonas* and phosphor-Ser protein in *Streptococcus pneumoniae*. Furthermore, *E. coli* EngA has been recently proposed to cycle between ribosomes and the membrane via interaction with the outer membrane protein OmpA<sup>772</sup>. *B. subtilis* possesses also a flagellar motor protein called MotB which contains a C-ter inner membrane domain of OmpA-type<sup>673,773</sup>. The possible interaction between EngA and YdiB and between EngA and this OmpA-like domain in MotB might explain the presence of YdiB near the cellular periphery in *B. subtilis*. (c.f. II.3.2.3 for more details)

The second conserved GTPase, EngB, beside its interaction with *B. subtilis* 50S and *S. aureus* 30S ribosomal proteins, contains the lysine comb suggested to mediate interaction with ribosomal RNA (c.f. II.3.2.3).

Recent result obtained by C. Grangeasse, our collaborator at IBCP (Lyon) confirmed the acetylase activity of YdiD in *S. pneumoniae*. This activity might be essential for the function of the ribosomal protein S18. The interaction between a highly flexible and basic N-ter of S18 with the Shine Dalgarno helix in mRNA is important for the formation of initiation complex during translation<sup>595</sup>. *B. subtilis* YdiD is in the same operon with YdiB and the phosphorylation of YdiD by YdiB possibly controls the acetylase activity of YdiD (c.f. II.3.1 and fig. 43).

The universally conserved YdiE of multifunction has recently been found to possess a conserved function: the universal t<sup>6</sup>A<sub>37</sub> tRNA modification which is found in all tRNAs pairing with Axx codons of mRNA in order to strengthen the A-U codon-anticodon interaction on the ribosome. Such modification is required for proper reading frame maintenance<sup>570</sup> and translocation from the A-site to the P-site, thus assuring the speed and accuracy of translation<sup>571</sup>. Mao & al.<sup>572</sup> reported a weak but significant ATPase activity of archaea *Methanococcus jannaschii* YgjD. Yeast YgjD was also found to possess ATPase activity which is indispensable for t<sup>6</sup>A modification<sup>569</sup>. Phosphorylation of YdiE by YdiB therefore might contribute to control this universal function of YdiE (c.f. II.3.1 and fig. 44).

We also propose that the gene downstream of *ydiB* encodes YdiC, the plausible cognate phosphatase of YdiB. Via the dephosphorylation of YdiB, EngA and YdiE by YdiC that was reported in this study, we suggest that YdiC can contribute to control the phosphorylating status of different substrates of YdiB.

Except for YdiE, the phosphorylation of the three other substrates by YdiB needs the presence of the activator polyamine. The effect of basic molecules on activity of YdiB as well as the summary of uptake, biosynthesis and export of polyamine can be found in II.2.3 and fig. 37. Most of cellular polyamines are found in complexes with RNA to simulate protein synthesis. Polyamines also induce *in vivo* assembly of 30S ribosomal subunits. Binding of polyamines to ribosomes resulted in increasing of accuracy of codon usage during protein synthesis, of +1 ribosomal frame-shift efficiency and facilitating translational read-through of mRNAs with the stop codon UAA. These ribosome-related functions of polyamine, which we propose here to be the activator of YdiB, are therefore in accordance with the plausible involvement of YdiB in ribosome function and/or biosynthesis.

Three other proteins which are involved in ribosome's function and whose biotides are phosphorylated by YdiB are also of particular interest for the future works: FusA (EF-G), InfA and TmrK.

Under oxidative stress condition, SodA, AhpF/C, YdiE and EngB are the four potential substrates of YdiB that possess different oxidative stress response activities:

It is worth noting that *E. coli yjeE*, (homologue of *ydiB*) is induced during norfloxacin treatment<sup>770</sup>. In 2007, Campbell & al.<sup>774</sup> discovered *E. coli rstA* as a multicopy suppressor of a conditional *yjeE* deletion strain. RstA is assumed to be the response regulator of the RstAB two-component signal transduction system (TCS)<sup>775</sup> and specifically phosphorylated by the autophosphorylating histidine kinase RstB<sup>776</sup>. *E. coli* double-deleted  $\Delta$ *rstAB* strain becomes hypersensitive towards ketoprofen<sup>777</sup>. The cellular role of RstAB is still obscure and the basis of this sensitivity is still unknown, but the toxicities of norfloxacin<sup>771</sup> and ketoprofen<sup>778,779</sup> are well-known to be related to oxidative stress. The double-deletion also activates translation of *rpoS* which encodes the  $\sigma^S$  subunit of RNA polymerase regulating general stress response genes in *E. coli*<sup>780</sup>. Like many other TCSs, the stimulus that triggers RstA is also still unknown. It was suggested that the depletion of YjeE might directly or indirectly activate RstA which then in turn, calls other genes required for overcoming the loss of *yjeE* into play<sup>774</sup>.

Under various oxidative stressful conditions, the cellular concentration of superoxide anion increases, putting cells under the risk of DNA, lipid and protein damages due to formation of hydroxyl radical. In this study, we provide evidence showing that YdiB, by phosphorylating and thus improving the superoxide scavenger efficiency of SodA, contributes to prevent cell death upon exposure to paraquat (*c.f.* II.4.1 & II.4.2).

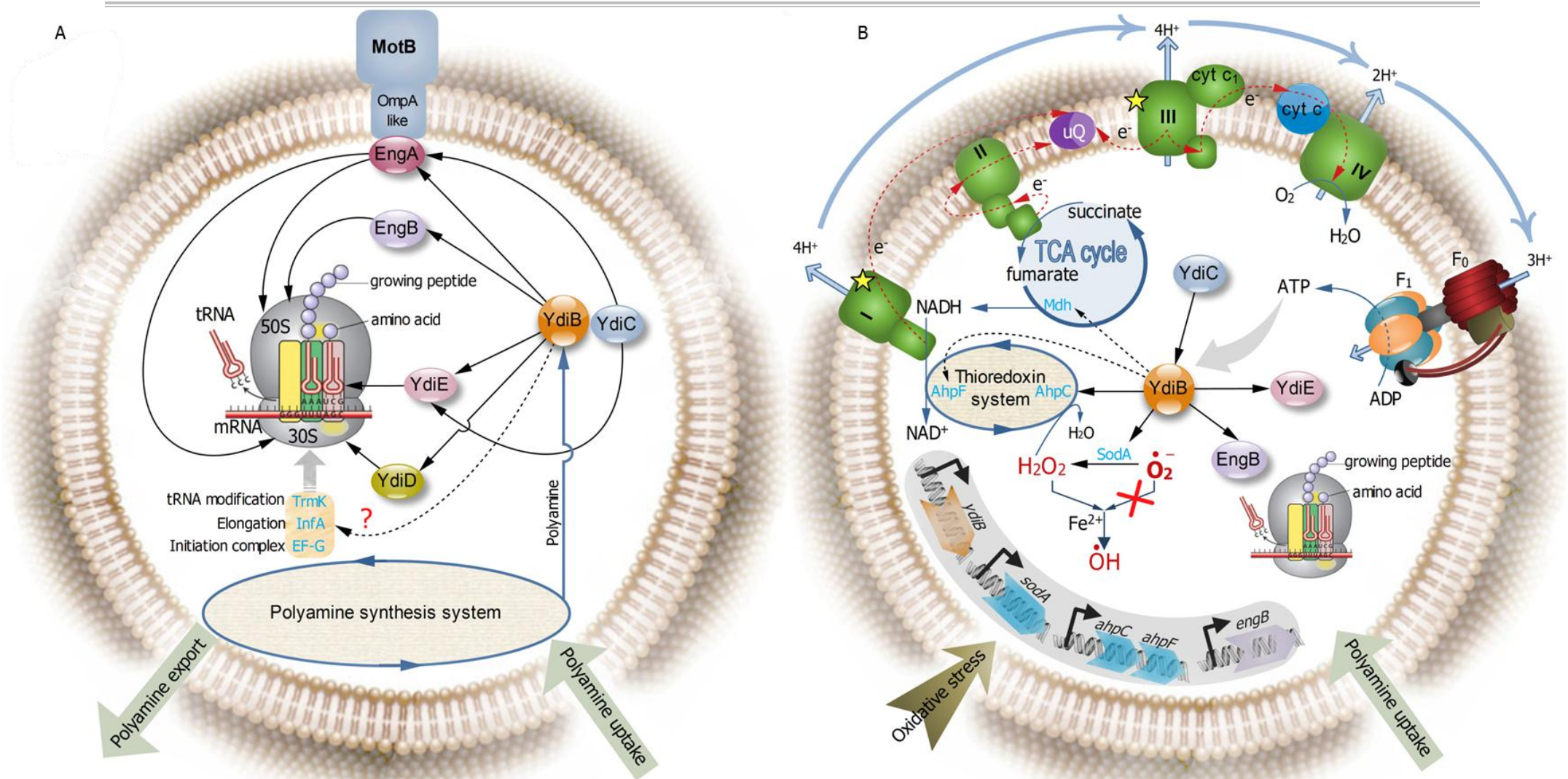
Not only *E. coli yjeE*, the genes *sodA*, *ahpC*, *ahpF*, *engB* of *B. subtilis* are also upregulated under oxidative stresses ( $H_2O_2$  and paraquat). Previous unpublished results in our group showed that SodA and the small subunit of alkyl hydroperoxide reductase AhpC are co-purified with YdiB in *B. subtilis*, suggesting their direct interaction *in vivo*. The roles of SodA, AhpC, AhpF in response to oxidative stress are quite well-established but that of EngB is still obscure. However, please recall that EngB is phosphorylated by YdiB in the presence of the activator polyamine. Interestingly, not only playing important role in protein synthesis, in the synthesis and function of ribosome, polyamines also participate in responding to oxidative stress. Spermidine and spermine as free radical scavengers conjunctionally function with SOD activity to reduce DNA breakage by oxygen radicals. *E. coli speABCDE* knock-out mutant, as putrecine and spermidine-deficient cells, could not survive in oxidative stress conditions that are non-toxic to wild-type cells. Polyamines therefore plausibly play a role in reducing toxic effects coming from oxidative stress, at least via activating kinase function of YdiB (*c.f.* in II.2.3 and the *fig.* 37).

In 2009, based on the feeding RNA interference method, Oberto & al.<sup>781</sup> showed that the *C. elegans* gene *osgl1* regulates paraquat induced oxidative stress in a *cep-1* dependent manner, where *osgl1* and *cep-1* are orthologues of *ydiE* and the transcriptional factor p53 respectively. In 2010, Katz & al.<sup>562</sup> provided evidence for the role of *E. coli ydiE (gcp)* in reducing the accumulation of glycated proteins-promoters of oxidative stress<sup>782-784</sup>. They proposed that, by binding to glycated proteins, YdiE facilitates the deglycating activity of "amadoriases", thus preventing further oxidative stress damage. The phosphorylation of YdiE by YdiB might play a role in regulating these oxidative stress-related activities of YdiE. Alternatively, under control of phosphorylation by YdiB (and/or YdiC), YdiE and EngB might regulate protein expression profile under oxidative stress condition, regarding to their ribosome-related functions previously mentioned. Interestingly, support to this hypothesis, the newest transcriptome data on *B. subtilis* released in 2012<sup>785</sup> reported that either *ydiB*, *ydiC*, *ydiD*, *ydiE*, *engA*, *engB* and *sodA* are induced under oxidative stress conditions, either by paraquat,  $H_2O_2$  or diamide treatments.

Along with SodA and AhpC/F, the malate dehydrogenase Mdh is also up-regulated upon oxidative stress<sup>786</sup> and its activity is required for protecting *E. coli* against  $H_2O_2$  or irradiation treatment<sup>729</sup>. These four proteins moreover were found to be Ser/Thr/Tyr phosphorylated *in vivo* not only in *B. subtilis* but also in other bacteria; and in this study we show that their biotides are phosphorylated by YdiB. Therefore, not only SodA, but AhpC/F and Mdh are also of particular interest for the future works regarding the oxidative stress-related function of YdiB (*c.f.* mini-review on oxidative stress in the *fig.* 51).

Given the special conservation pattern of YdiB which is exclusively conserved in bacteria but not in eukaryote, this protein of unknown function is in the list of development of new therapeutic drugs, such as a new generation of antibiotics<sup>787,788</sup>. The prerequisite step towards this final goal therefore is to understand its cellular function as well as its molecular mechanism. The finding of this kinase activity, which possibly plays a role in ribosome function in normal physiological condition and in protecting cells from oxidative stress damage; as well as insights into its molecular mechanism involving oligomerization status and basic molecule effect, therefore undoubtedly contribute a step towards comprehensive understanding of this attractive but previously poorly characterized protein.

In term of fundamental biology, along with the five major well-known prokaryotic protein kinase families, (including Histidine kinases in Two-component systems, IDHK/P and HPrK/P discovered in the 1980's, eukaryotic like-Ser/Thr kinases of Hank's type in the 1990s'; and the recently discovered branch of BYks in the 2000's), the discovery of YdiB-like family possessing dual Ser/Thr and Tyr protein kinase activity in the 2010's therefore open a new chapter on prokaryotic protein kinases. Given the fact that this new family is broadly conserved in bacterial kingdom, we propose to name it Ubk for "Ubiquitous bacterial kinase".



**Figure 54:** Working models of YdiB's cellular function(s) in *B. subtilis* during normal and oxidative stress conditions

**A:** Possible involvement of YdiB in biosynthesis and/ or function of ribosome under normal condition. Phosphorylation of EngA, EngB, YdiD and YdiE by YdiB possibly regulates ribosomal-related activities of these substrates. Except for YdiE, the phosphorylation of the three other substrates by YdiB needs the presence of activator polyamine. YdiC is the possible cognate phosphatase of YdiB which can contribute to control the phosphorylating status of these substrates. Three other proteins which are probably involved in ribosome's function and whose biotides are phosphorylated by YdiB are indicated in cyan color.

**B:** Involvement of YdiB in response to oxidative stress. Under various oxidative stressful conditions, the cellular concentration of superoxide anion increases, putting cells under the risk of DNA, lipid and protein damages due to formation of hydroxyl radical. YdiB, by phosphorylating and thus improving the superoxide scavenger efficiency of SodA, contributes to prevent cell death. Malate dehydrogenase (Mdh) and Alkyl hydroperoxide reductase (AhpC/AhpF), which were shown to protect cells from  $H_2O_2$  stress, are phosphoproteins *in vivo* and their biotides are phosphorylated by YdiB *in vitro*. The genes *sodA*, *ahpC*, *ahpF*, *engB* of *B. subtilis* are upregulated under  $H_2O_2$  and paraquat stresses and *E. coli yjeE*, (homologue of *ydiB*) is induced during norfloxacin treatment. Not only EngB, YdiE also plays role during oxidative stress. These two latter potential substrates of YdiB (as showed in A) therefore might control expression of *B. subtilis* proteins during oxidative stress. The dynamic reversible response process is furthermore possibly controlled by YdiC, the cognate phosphatase of YdiB

*Material  
&  
Methods*

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**MATERIAL-METHODS****General methods**

*E. coli* and *B. subtilis* strains were grown in LB (Luria–Bertani) medium. Antibiotics were used at concentration of 100 µg/mL for ampicillin; 150 µg/mL for spectinomycin.

Polymerase enzyme Phusion, T4 DNA ligase and other restriction enzymes were obtained from Finnzymes, Fermentas and New England Biolabs. Gel extraction kits and plasmid midi-prep kits were from QBiogene.

ATP- $\gamma$ -S, PNBM (*i.e.* *p*-nitrobenzyl mesylate) and anti-thiophosphate ester specific rabbit monoclonal antibody were purchased from Epitomic. Immobilon-P transfer membranes were from Millipore. Donkey anti-rabbit antibody conjugated to horseradish peroxidase was purchased from Amersham. Western chemiluminescence reagents were obtained from Pierce.

Radioactive ATP- $\gamma$ -<sup>32</sup>P and Super resolution phosphor screen were obtained from Perkin Elmer.

Biotides were synthesized by JPT Peptide Technologies. SAM2 biotin capture membrane was purchased from Promega.

All other compounds were from Sigma-Aldrich.

**Molecular biology methods**

## Plasmids and strains construction

Bacterial strains and plasmids used in this study are listed in *tab.* 10.

The *B. subtilis*  $\Delta$  *ydiB* deletion mutant was previously constructed by Karst & al.<sup>10</sup>

The *B. subtilis* strain resistant to spectinomycin was obtained by MH Laaberki by transforming pDR111 into *B. subtilis* WT 168. The plasmid pDR111 created by David Rudner (Harvard Medical School) is a derivative of *Pspac-hy* plasmid pJQ43<sup>789</sup> with Spec<sup>R</sup> cassette resistance.

Lewis & al.<sup>578</sup> developed a series of plasmid vectors allowing fusions of *gfp* to target gene. One of these vectors was then used to create *B. subtilis* strain mutant 1049 where the gene *rspB* encoding small-subunit ribosomal protein S2 was fused to *gfp*. We obtain this strain 1049 as a generous gift from Pr. Errington (Sir William Dunn School of Pathology, University of Oxford).

**Table 10: Strains and plasmids used in this study**

Strains and plasmids	Genotype	Source or reference
<b><i>Bacillus subtilis</i></b>		
WT 168	<i>trpC2</i>	Institute Pasteur
WT-specR	<i>trpC2 amyE::(spc P<sub>SPANK-HY</sub> lacI)</i>	This work, by MH Laaberki
$\Delta$ <i>ydiB</i> -specR	<i>trpC2 ydiB::(spc)</i>	Karst & al. <sup>10</sup>
RpsB-GFP (1049)	<i>trpC2 amyE::(spc P<sub>Xyl</sub>-rpsB-gfp)</i>	Gift from Pr. Errington <sup>a</sup>
<b><i>Escherichia coli</i></b>		
BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm <math>\lambda</math>(DE3)</i>	Novagen <sup>®</sup>
JM109	e14 <sup>-</sup> (McrA <sup>-</sup> ) <i>recA1 endA1 relA1 gyrA96 thi-1 hsdR1.7 (r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>) supE44 <math>\Delta</math> (lac-proAB) [F' <i>traD36 proAB lacI<sup>a</sup> Z<math>\Delta</math>M15</i>]</i>	Stratagene <sup>®</sup>
<b>Plasmids</b>		
pDR111	<i>amyE::(spc P<sub>SPANK-HY</sub> lacI)</i>	David Rudner (unpublished) <sup>b</sup>
pREP4	<i>lacI (kan)</i>	Qiagen
pGEX-4T1	<i>bla P<sub>TAC</sub> gst lacI</i>	Amersham GE Healthcare
pGEX-4T1-gst-ydiB	<i>bla P<sub>TAC</sub> gst-ydiB lacI</i>	This work, by J. Karst
pET15b	<i>bla P<sub>T7</sub> his<sub>6</sub> lacI</i>	Novagen
pET15b-his <sub>6</sub> -ydiB	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiB</i>	Karst & al. <sup>10</sup>
pET15b-his <sub>6</sub> -ydiB-K41A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBK41A-lacI</i>	Karst & al. <sup>10</sup>
pET15b-his <sub>6</sub> -ydiB-K41R	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBK41R-lacI</i>	Karst & al. <sup>10</sup>
pET15b-his <sub>6</sub> -ydiB-T42S	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBT42S-lacI</i>	Karst & al. <sup>10</sup>
pET15b-his <sub>6</sub> -ydiB-S60A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBS60A-lacI</i>	This work
pET15b-his <sub>6</sub> -ydiB-T62A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBT62A-lacI</i>	This work
pET15b-his <sub>6</sub> -ydiB-H78A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBH78A-lacI</i>	This work
pET15b-his <sub>6</sub> -ydiB-D80A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBD80A-lacI</i>	Karst & al. <sup>10</sup>
pET15b-his <sub>6</sub> -ydiB-Y82A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBY82A-lacI</i>	This work
pET15b-his <sub>6</sub> -ydiB-R83A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBR83A-lacI</i>	This work
pET15b-his <sub>6</sub> -ydiB-E106A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBE106A-lacI</i>	Karst & al. <sup>10</sup>
pET15b-his <sub>6</sub> -ydiB-E106D	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBE106D-lacI</i>	Karst & al. <sup>10</sup>
pET15b-his <sub>6</sub> -ydiB-W107A	<i>bla P<sub>T7</sub> his<sub>6</sub> ydiBW107A-lacI</i>	This work
pET28a <sup>+</sup>	<i>kan P<sub>T7</sub> his<sub>6</sub> lacI</i>	Novagen
pET28a <sup>+</sup> -gst	<i>kan P<sub>T7</sub> gst his<sub>6</sub> lacI</i>	This work, C. Wicker-Planquart
pET28a <sup>+</sup> -gst-ydiB-his <sub>6</sub>	<i>kan P<sub>T7</sub> gst-ydiB- his<sub>6</sub>- lacI</i>	This work, by J. Karst

pET15b-his <sub>6</sub> -engA	<i>bla</i> P <sub>17</sub> <i>his</i> <sub>6</sub> <i>engA</i>	Schaefer & al. <sup>790</sup>
pET15b-his <sub>6</sub> -engA-GD2KH	<i>bla</i> P <sub>17</sub> <i>his</i> <sub>6</sub> <i>engA-GD2KH</i>	Foucher & al. <sup>639</sup>
pET15b-his <sub>6</sub> -engB	<i>bla</i> P <sub>17</sub> <i>his</i> <sub>6</sub> <i>engB</i>	Schaefer & al. <sup>790</sup>
pET21b <sup>+</sup>	<i>bla</i> P <sub>17</sub> <i>his</i> <sub>6</sub> <i>lacI</i>	Novagen
pET21b <sup>+</sup> -ydiC-his <sub>6</sub>	<i>bla</i> P <sub>17</sub> <i>ydiC-lacI</i>	This work, by B. Hermant
pET21b <sup>+</sup> -ydiD-his <sub>6</sub>	<i>bla</i> P <sub>17</sub> <i>ydiD-lacI</i>	This work, by B. Hermant
pBAD24	<i>ori</i> pBR322; <i>bla</i> P <sub>BAD</sub>	Guzman & al. <sup>791</sup>
pBAD24-his <sub>6</sub>	<i>ori</i> pBR322; <i>bla</i> P <sub>BAD</sub> <i>his</i> <sub>6</sub>	This work, by MH. Laaberki
pBAD24-ydiE-his <sub>6</sub>	<i>bla</i> P <sub>BAD</sub> <i>ydiE-his</i> <sub>6</sub>	This work, by MH. Laaberki
pBAD24-sodA-his <sub>6</sub>	<i>bla</i> P <sub>BAD</sub> <i>sodA-his</i> <sub>6</sub>	This work, by MH. Laaberki

a: Pr. Errington (Sir William Dunn School of Pathology, University of Oxford); b: David Rudner (Harvard Medical School)

For production of His<sub>6</sub>-YdiB, GST-YdiB and His<sub>6</sub>-YdiB-GST, the gene *ydiB* was amplified from *B. subtilis* genomic DNA by PCR using primers (1, 2); and (3, 4) respectively (tab. 11). The PCR products were then cut by restriction enzymes (tab. 10) before being ligated into pre-digested pET15b and resulting pET15b-his<sub>6</sub>-ydiB and pGEX-4T1-gst-ydiB plasmids respectively (tab. 10).

The plasmid pET15b-his<sub>6</sub>-ydiB was then used as template to create different N-ter His<sub>6</sub> tagged single site-directed protein-mutants of conserved residues using the "QuickChange Site-Directed Mutagenesis" from Stratagene and primers 5 to 28 from the tab. 11.

**Table 11: Primers and restriction enzymes used in this study**

N°	Primers	Sequences	Restriction enzymes
<b>Cloning YdiB and its single site directed mutants</b>			
1	ydiB-f (→ pET15b)	GGGCATATGGTGAAGCAATTTAAAATGGAGAAC	<i>NdeI</i>
2	ydiB-r (→ pET15b)	GGGGCTCAGCTCAATTGCTAATATTGTCATGTCTAC	<i>BlpI</i>
3	ydiB-f (→ pGEX4T1)	GGGGAATTCGTGAAGCAATTTAAAATGGAGAAC	<i>EcoRI</i>
4	ydiB-r (→ pGEX4T1)	GGGCTCGAGATTGCTAATATTGTCATGTCTACT	<i>XhoI</i>
5	ydiB-K41A-f (→ pET15b-his <sub>6</sub> -ydiB)	GGGCGATTTAGGTGCCGGCCCAACGACTTTTACGAAAGG	<i>NaeI</i>
6	ydiB-K41A-r (→ pET15b-his <sub>6</sub> -ydiB)	CCTTTCGTAAGAGTCGTTGCCGGCCACCTAAATCGCCC	<i>NaeI</i>
7	ydiB-K41R-f (→ pET15b-his <sub>6</sub> -ydiB)	GATTTAGGTGCCGGCAGAAGCAGCTTTTACGAAAGGTTTTGC	<i>NaeI</i>
8	ydiB-K41R-r (→ pET15b-his <sub>6</sub> -ydiB)	GCAAAACCTTTTCGTAAGAGTCGTTCTGCCGGCACCTAAATC	<i>NaeI</i>
9	ydiB-T42S-f (→ pET15b-his <sub>6</sub> -ydiB)	GATTTAGGTGCCGGCAAATTCGACTTTTACGAAAGGTTTTGC	<i>NaeI</i>
10	ydiB-T42S-r (→ pET15b-his <sub>6</sub> -ydiB)	GCAAAACCTTTTCGTAAGAGTCGATTTGCCGGCACCTAAATC	<i>NaeI</i>
11	ydiB-S60A-f (→ pET15b-his <sub>6</sub> -ydiB)	GGAATTACACGTATTGTTAACGCTCCGACTTTTACAATTATAAAAG	<i>HpaI</i>
12	ydiB-S60A-r (→ pET15b-his <sub>6</sub> -ydiB)	CTTTTATAATTGTAAGAGTCGGAGCGTTAACAAATACGTGTAATTC	<i>HpaI</i>
13	ydiB-T62A-f (→ pET15b-his <sub>6</sub> -ydiB)	CGTATTGTTAACAGTCCGGCTTTTACAATTATAAAAG	<i>HpaI</i>
14	ydiB-T62A-r (→ pET15b-his <sub>6</sub> -ydiB)	CTTTTATAATTGTAAGAGCCGGACTGTTAACAAATACG	<i>HpaI</i>
15	ydiB-H78A-f (→ pET15b-his <sub>6</sub> -ydiB)	GGCGTACTTCTCTTTATGCCATGGAGCTCTATAGAATG	<i>AatII</i>
16	ydiB-H78A-r (→ pET15b-his <sub>6</sub> -ydiB)	CATTCTATAGACTCCATGGCATAAAGAGGAAGTACGCC	<i>AatII</i>
17	ydiB-D80A-f (→ pET15b-his <sub>6</sub> -ydiB)	GGCGTACTTCTCTTTATCATATGGCTGTGTATAGAATGGAAGATG	<i>NdeI</i>
18	ydiB-D80A-r (→ pET15b-his <sub>6</sub> -ydiB)	CATCTTCCATTCTATACAGCCATATGATAAAGAGGAAGTACGCC	<i>NdeI</i>
19	ydiB-Y82A-f (→ pET15b-his <sub>6</sub> -ydiB)	CTTTATCATATGGATGTGGCTAGAATGGAAGATGAAAG	<i>NdeI</i>
20	ydiB-Y82A-r (→ pET15b-his <sub>6</sub> -ydiB)	CTTTTCATCTCCATTCTAGCCACATCCATATGATAAAG	<i>NdeI</i>
21	ydiB-R83A-f (→ pET15b-his <sub>6</sub> -ydiB)	CTTTATCATATGGATGTGTATGCAATGGAAGATGAAAG	<i>NdeI</i>
22	ydiB-R83A-r (→ pET15b-his <sub>6</sub> -ydiB)	CTTTTCATCTCCATTGCATACACATCCATATGATAAAG	<i>NdeI</i>
23	ydiB-E106A-f (→ pET15b-his <sub>6</sub> -ydiB)	GGTGTCTGTCTCGTTGCATGGGCCCATTTAATTGAAGAAC	<i>ApaI</i>
24	ydiB-E106A-r (→ pET15b-his <sub>6</sub> -ydiB)	GTTCTTCAATTAATGGGCCCATGCAACGAGACAGACACC	<i>ApaI</i>
25	ydiB-E106D-f (→ pET15b-his <sub>6</sub> -ydiB)	CAAGGTGTCTGTCTGTCGACTGGGCTCATTTAATTGAAG	<i>SalI</i>
26	ydiB-E106D-r (→ pET15b-his <sub>6</sub> -ydiB)	CTTCAATTAATGAGCCCATGTCGACGAGACAGACACCTTG	<i>SalI</i>
27	ydiB-W107A-f (→ pET15b-his <sub>6</sub> -ydiB)	GGTGTCTGTCTCGTTGAAGCGGCCCATTTAATTGAAGAACAG	<i>HaeIII</i>
28	ydiB-W107A-r (→ pET15b-his <sub>6</sub> -ydiB)	CTGTTCTTCAATTAATGGGCCCTTCAACGAGACAGACACC	<i>HaeIII</i>
<b>Cloning potential substrates of YdiB</b>			
29	ydiC-f (→ pET21b <sup>+</sup> )	GCCCATATGATGACAATATTAGCAATTGATAC	<i>NdeI</i>
30	ydiC-r (→ pET21b <sup>+</sup> )	CTCGAGCTTTTGACTTTCAATCCATTTTCGCTTCAGC	<i>XhoI</i>
31	ydiD-f (→ pET21b <sup>+</sup> )	CATATGATGAAAACAAAAGCAGCGGTGAGAAATATGCGGTGAG	<i>NdeI</i>
32	ydiD-r (→ pET21b <sup>+</sup> )	CCGCTCGAGCTCATTATTCGTACCCACATAAATTAACGC	<i>XhoI</i>
33	ydiE-f (→ pBAD24-his <sub>6</sub> )	GCGCCCATGGGTGAGCAAAAAGACATGTACG	<i>NcoI</i>
34	ydiE-r (→ pBAD24-his <sub>6</sub> )	GCGCGTCGACTCTCGTGAGACTTTGATAAGAAGTC	<i>SalI</i>
35	sodA-r (→ pBAD24-his <sub>6</sub> )	GCGCCCATGGCTTACGAACTTCCAGAATTACCTTATGCG	<i>NcoI</i>
36	sodA-f (→ pBAD24-his <sub>6</sub> )	GCGCGTCGACTTTTGTCTCGCTGTATAGACG	<i>SalI</i>
37	engA-f (→ pET15b)	GGGCATATGGGTAAACCTGTCGTAGCCATTGTC	<i>NdeI</i>
38	engA-r (→ pET15b)	GGGGCTCAGCTTATTTTCTAGCTCTTGCAAATAT	<i>BlpI</i>
39	engA-GD2KH-f (→ pET15b)	CATATGGAAAGTTATCAATTCTGTCTGATC	<i>NdeI</i>
40	engB-f (→ pET15b)	GGAATTCATATGAAAGTCAAAAGTCAGAAATCGT	<i>NdeI</i>
41	engB-r (→ pET15b)	ACTGAAGCTCAGCTACCGGTTTATCATTTTTTTGATCGC	<i>BlpI</i>

The plasmid pGEX-4T1-gst-ydiB was then digested by BamHI and XhoI and *ydiB*-containing insert was then ligated into BamHI and XhoI pre-digested pET28a<sup>+</sup>-gst, a derivative from pET28a<sup>+</sup> previously created by C. Wicker-Planquart, resulting in the plasmid pET28a<sup>+</sup>-gst-ydiB-his<sub>6</sub> where *ydiB* is double tagged (*tab.* 10).

The primers (29, 30) and (30, 31) (*tab.* 11) were used to amplify *ydiC* and *ydiD* respectively with *B. subtilis* genomic DNA as template for PCR. The PCR products were then cut by NdeI and XhoI before being ligated into pre-digested pET21b<sup>+</sup> to have pET21b<sup>+</sup>-ydiC-his<sub>6</sub> and pET21b<sup>+</sup>-ydiD-his<sub>6</sub> plasmids (*tab.* 11) which will be used for purification of C-ter His<sub>6</sub> tagged YdiC and YdiD.

The primers (33, 34) and (35, 36) (*tab.* 11) were used to amplify *ydiE* and *sodA* respectively with *B. subtilis* genomic DNA as template for PCR. The PCR products were then cut by NcoI and SalI before being ligated into pBAD24-his<sub>6</sub> pre-digested by the same enzymes. This plasmid created by MH Laaberki, is a derivative from pBAD24 where the SphI site was replaced with an insert coding for His<sub>6</sub> tag following an NdeI site. The resulting pBAD24-ydiE-his<sub>6</sub> and pBAD24-sodA-his<sub>6</sub> (*tab.* 10) were used for purification of C-ter His<sub>6</sub> tagged YdiE and SodA respectively.

The primers (37, 38); (39, 38) and (40, 41) (*tab.* 11) were used to amplify *engA*; the GD2KH domain of *engA* and *engB* respectively with *B. subtilis* genomic DNA as template for PCR. After being cut by different restriction enzymes (*tab.* 11), the digested products were then ligated into pre-digested pET15b. The resulting pET15b-his<sub>6</sub>-engA, pET15b-his<sub>6</sub>-engA-GD2KH and pET15b-his<sub>6</sub>-engB (*tab.* 10) were used for purification of N-ter His<sub>6</sub> tagged EngA, EngA-GD2KH domain and EngB respectively.

The contribution of different people for the construction of strains and plasmids used in this study was highlighted in the *tab.* 10.

#### Competent cells and plasmid transformation

Heat shock plasmid transformation into *E. coli* competent cells prepared by calcium chloride method were performed as described by Tu & al.<sup>792</sup>.

The plasmids pET15b-his<sub>6</sub>-ydiB; pET21b<sup>+</sup>-ydiC-his<sub>6</sub>; pET21b<sup>+</sup>-ydiD-his<sub>6</sub>; pBAD24-sodA-his<sub>6</sub>; pET15b-his<sub>6</sub>-engA; pET15b-his<sub>6</sub>-engA-GD2KH and pET15b-his<sub>6</sub>-engB were separately transformed into *E. coli* BL21 (DE3). The plasmids pBAD24-ydiE-his<sub>6</sub> was transformed into *E. coli* BL21 (DE3) pre-transformed with pREP4 and the plasmid pGEX-4T1-gst-ydiB was transformed into *E. coli* JM109.

Selection of bacteria carrying recombinant plasmids was performed on LB agar plates containing ampicillin. Positive clones were confirmed by DNA sequencing (Genome Express).

#### Biochemistry methods

##### Recombinant protein over-expression and purification

Positive clone was seeded into 1 L of ampicillin containing LB medium. Sterile glucose was used to reduce basal expression levels in pET<sup>793</sup> (1% w/v), pGEX<sup>794</sup> (2% w/v) and pBAD<sup>795</sup> (0.1% w/v) systems via catabolite repression mechanism. Cultures were grown at 37 °C until the OD<sub>600</sub> reached 0.6 - 0.8 then 1 mM IPTG (with pET system) or 0.2 % (w/v) arabinose (with pBAD system) were added for induction during 4h at 37°C. Cells were harvested by centrifugation, and re-suspended in 20 ml of appropriate lysis buffer (*tab.* 12).

The lysate was disrupted twice at 18000 p.s.i. or fourth times at 10500 p.s.i in a microfluidizer apparatus, cell debris was pelleted by centrifugation at 30000 g for 30 min at 4 °C and clarified lysate was collected.

*For His<sub>6</sub>-YdiB and its 12 mutants purification:* the clarified lysate in lysis buffer 1 was first purified by an anion-exchange step. 10 ml DEAE-cellulose slurry (Sigma) pre-equilibrated in lysis buffer was added to each clear lysate and the solution was mixed gently at 4 °C for 60 min. The resin was then washed three times with a washing buffer 1 and the beads were re-suspended in 20 mL of elution buffer 1 (*tab.* 12), incubated for 30 min at 4 °C then loaded onto nickel column pre-equilibrated with lysis buffer 2. The next step of Ni-NTA purification is described below.

*For YdiC-His<sub>6</sub>, YdiD-His<sub>6</sub>, YdiE-His<sub>6</sub>, SodA-His<sub>6</sub> purification:* the clarified lysate in lysis buffer 2 was loaded onto a nickel chelate chromatography column (Qiagen) pre-equilibrated in lysis buffer 2 at 4°C. The column (Econo-pac from Bio-Rad) was allowed to drain, washed with 100 mL of washing buffer 2 and eluted with 10 - 15 mL of elution buffer 2 (*tab.* 12).

*For GST-YdiB purification:* the clarified lysate in lysis buffer 3 was loaded onto 4 mL glutathione-sepharose column (Sigma) pre-equilibrated in lysis buffer 3 and the protein-resin slurry was mixed gently at 4 °C for 60 min. The column was allowed to drain, washed with 60 mL of washing buffer 3 and eluted with 10 ÷ 15 mL of elution buffer 3 (*tab.* 12).

The purity of the recombinant proteins was analyzed by 14% polyacrylamide gel electrophoresis and stained with Coomassie blue or electro-blotted onto Immobilon-P transfer membranes for Western blotting analysis. To detect His-tagged or GST-fusion proteins, the Super-Signal West Pico kit (Pierce) was used along with 1<sup>st</sup> antibodies against His-tag (HisProbe-HPR [*i.e.* horseradish peroxidase]; Pierce) or GST (anti-GST-HPR conjugate; Euromedex), as described by the manufacturer. In some cases, a size-exclusion chromatography step was added to increase the protein purity or to separate monomeric and oligomeric forms (see below).



The protein concentration was determined by the Bradford protein assay (Coomassie plus, Pierce), using BSA as a standard. Purified proteins were concentrated by vivaspin concentrators (cut-off 10 kDa, Amersham GE Healthcare), aliquoted and immediately frozen in liquid nitrogen and kept at -80°C until use.

Before use, the thawed protein was centrifuged for 15 min at 14000 g at 4 °C to eliminate aggregates and the supernatant was loaded onto PD SpinTrap G-25 column (Amersham GE Healthcare) pre-equilibrated with the appropriate buffer according to each assay.

### Size-exclusion chromatography

Size-exclusion chromatography experiments were performed at 4 °C on a Superdex 75 10/300 GL column (Amersham GE Healthcare), equilibrated with buffer 1 or 2 of high or low salt concentration respectively (*tab.* 12). The samples were centrifuged for 15 min at 14000 g at 4 °C prior loading 250 µL aliquots onto the column at a protein concentration ~2.5 mg/mL. Elution profiles were monitored by recording the absorbance at 280nm using a flow rate of 1 mL/min. The Gel filtration calibration kit LMW from Amersham GE Healthcare was used to determine approximately the molecular mass of each protein.

Fractions corresponding to monomeric and oligomeric forms of His<sub>6</sub>-YdiB were collected separately for studying the effect of oligomerization on kinase activity.

**Table 12: Buffers used in this study**

Buffers	Composition
<i>Protein purification</i>	
Lysis buffer 1	50 mM HEPES/KOH pH 7.5, 10 mM NaCl, 5 mM β-ME supplemented with 1 mM PMSF every 20 min. and Protease inhibitor cocktail tablets from Roche
Lysis buffer 2	50 mM Tris/HCl pH 8, 250 mM NaCl, 20 mM imidazole, 5 mM β-ME supplemented with 1 mM PMSF every 20 min. and Protease inhibitor cocktail tablets.
Lysis buffer 3	10 mM Na <sub>2</sub> HPO <sub>4</sub> , 150 mM NaCl, 10% glycerol, 5 mM β-ME supplemented with 1 mM PMSF every 20 min. and Protease inhibitor cocktail tablets
Washing buffer 1	50 mM HEPES/KOH pH 7.5, 50 mM NaCl, 5 mM β-ME supplemented with 1 mM PMSF every 20 min.
Washing buffer 2	50 mM HEPES/ KOH pH 7.5, 300 mM NaCl, 20 mM imidazole, 5 mM β-ME supplemented with 1 mM PMSF every 20 min.
Washing buffer 3	50 mM HEPES/KOH pH 7.5, 200 mM NaCl, 0.5 % Tween 20, 5 mM β-ME supplemented with 1 mM PMSF every 20 min.
Elution buffer 1	50 mM HEPES/KOH pH 7.5, 500 mM NaCl, 5 mM β-ME
Elution buffer 2	50 mM HEPES/KOH pH 7.5, 100 mM NaCl, 250 mM imidazole, 10% glycerol, 5 mM β-ME
Elution buffer 3	50 mM Tris/HCl pH 7.5, 10 mM reduced glutathion, 10% glycerol
<i>Size-eclusion chromatography</i>	
Equilib. buffer 1	50 mM HEPES/KOH pH 7.5, 500 mM NaCl
Equilib. buffer 2	50 mM HEPES/KOH pH 7.5, 50 mM NaCl
<i>Ribosome and ribosomal subunits purification</i>	
Washing buffer 4	10 mM Tris/HCl pH 7.5, 30 mM KCl, 100 mM Mg(CH <sub>3</sub> COO) <sub>2</sub> , 5 mM β-ME, 1 mM DTT
Lysis buffer 4	Washing buffer 4 with 500U DNase I RNase-free, Protease inhibitor cocktail tablets
Sucrose gradient	10 mM Tris/HCl pH 7.5, 50 mM NH <sub>4</sub> Cl, 10 mM MgCl <sub>2</sub> , 1 mM DTT, 5-40% sucrose
<i>SDS-PAGE and Western blotting</i>	
Laemmli 4x	500 mM Tris/HCl pH 6.8, 20% SDS, 40% glycerol, 1% Bromophenol Blue, 5 mM β-ME
Coomassie stain	40% Ethanol, 10% CH <sub>3</sub> COOH, 1.5% w/v R250 coomassie from Roth
Distain solution	30% Ethanol, 10% CH <sub>3</sub> COOH
TBS buffer	10 mM Tris/HCl pH 7.5, 0.137 M NaCl
TBST buffer	TBS, 0.05% w/v Tween20
Blocking buffer	TBST, 5% w/v non-fat powdered milk
<i>Other buffers/solvents</i>	
Kinase buffer 1	25 mM Tris/HCl pH 7.4, 0.5 mM MgCl <sub>2</sub> , 1 mM DTT
Kinase buffer 2	25 mM Tris/HCl pH 7.4, 10 mM MgCl <sub>2</sub>
Stopping buffer	100 mM EDTA, 4 M NaCl
pNPP buffer	50 mM MES pH 5.0, 5 mM MgCl <sub>2</sub>
TLC solvent 1	Ethanol/acetic acid/water (1:1:1, v/v/v)
TLC solvent 2	2-methyl-1-propanol (isobutyl alcohol)/formic acid/water (8:3:4, v/v/v)
TLC stain	0.5% ninhydrin in acetone
Fixing solution	4% v/v paraformaldehyde, 30 mM Na <sub>2</sub> SO <sub>4</sub> pH 7.5
Saturation buffer	PBS, 5% milk

**Abbreviations** HEPES: **H**ydroxy**e**thyl **p**iperazine**e**thanesulfonic acid, β-ME: β-**M**ercapto**e**thanol, PMSF: **P**henyl**m**ethylsulfonyl **f**luoride, DTT: **D**ithio**t**hreitol, Tris: **T**ris-(hydroxymethyl)-aminomethane, TBS: Tris-buffered saline, TLC: **T**hin **l**ayer **c**hromatography

### Ribosome purification

Highly purified ribosomes and ribosomal subunits were prepared from *B. subtilis* (strain WT 168, *tab.* 10) as described by Daigle and Brown<sup>796</sup> using centrifugations over sucrose cushions and gradients. Cultures were grown at 37 °C until the OD<sub>600</sub> reached 0.6 - 0.8. Cells were harvested by centrifugation and washed with washing buffer 4 before being re-suspended in lysis buffer 4 (*tab.* 12). The lysate was microfluidized three times at 10000 p.s.i., before being centrifuged at 15000 g for 30 min. at 4 °C.

The ribosome-containing supernatant was recovered and then layered onto a 5-40 % continuous sucrose gradient (*tab.* 12) followed by centrifugation at 200000 g for 16 h at 4 °C. Fractions of 400 µL were collected and identification of different ribosomal subunit-containing fractions was monitored by absorbance at 260 nm. The fractions corresponding to the small or large subunit were gathered and quantification of intact ribosome or ribosomal subunits was determined as follow: 1 A260 unit is equivalent to 23; 34 or 69 pmol of 70S, 50S and 30S, respectively.

### Protein co-purification with GST-YdiB-His<sub>6</sub>

Purified GST-YdiB-(His)<sub>6</sub> and the control GST-(His)<sub>6</sub>, as the bait proteins, were incubated alone or with immobilized glutathione resin. Captured bait proteins were then incubated with *B. subtilis* lysate containing putative prey proteins. After washing to remove unbound proteins, retained components were eluted and separated by SDS-PAGE. Several additional bands were revealed in case of GST-YdiB-(His)<sub>6</sub> but not with the control GST-(His)<sub>6</sub>. These bands containing potential partners of YdiB were then identified by mass spectrometry. This work had been done by J. Karst before my arrival to IBS and thus will be introduced only in the supplementary data.

### Kinase assay

Thanks to the recent commercialization of ATP-γ-S, PNBM and anti-thiophosphate ester antibody by Epitomic, the kinase assays were performed not only by classical radioactive method but also by western blotting.

#### *Kinase assay using radioactive ATP-γ-<sup>32</sup>P*

Characterization of autophosphorylation activity and MBP-phosphorylation activity of YdiB and of protein-mutants; as well as phosphorylation of YdiD, EngA, EngB and ribosomal 30S and 70S by YdiB were carried out by using radioactive ATP-γ-<sup>32</sup>P.

5 µM of each recombinant protein or ~20 µg of ribosome or ribosomal 30S were incubated in kinase buffer 1 (*tab.* 11) with 5 µCi γ<sup>32</sup>-ATP in the absence or presence of 5 µM YdiB, with or without 0.1 µM of poly-L-lysine during 10 minutes at 30°C in the total reaction volume of 20 µL. Laemmli 4x buffer (*tab.* 12) was added to stop the reaction and the mixture was heated at 100 °C for several minutes before being submitted to SDS-PAGE.

After electrophoresis in denaturing condition, the gel containing reaction components was first boiled in TCA 16% w/v during 15 min. in order to eliminate polyphosphates and hydrolyze phosphorylated nucleic acids. The gel afterwards was stained by coomassie (*tab.* 12) and allowed to dry using Gel drying kit (Promega) before being submitted to autoradiography using Super resolution phosphor screen and Cyclon Plus PhosphoImager (PerkinElmer) for visualization. When indicated, the quantification of radioactive signal correlated with incorporated phosphate was performed by ImageJ software.

#### *Kinase assay using ATP-γ-S*

In 2007, Allen & al.<sup>797</sup> developed an innovative method for labeling kinase substrates based on ATP-γ-S as phosphate donor. Once accepted this phosphate donor, the substrate becomes thiophosphorylated and could be alkylated by PNBM to yield thiophosphate esters which is then specifically recognized by anti-thiophosphate ester antibody. The products were then commercialized by Epitomic in 2010. The evident advantage of this method lies in using of nonradioactive material, although it is less sensitive than the classical method and thus requires using more materials for setting up kinase reaction.

Phosphorylation of YdiE, SodA and ribosomal 50S subunit by YdiB was obtained using ATP-γ-S.

30 µM of each protein or ~20 µg of ribosomal 50S were incubated in kinase buffer 2 (*tab.* 12) with 500 µM ATP-γ-S in the absence or presence of 15 µg YdiB at 30°C for different times depending on each assay. After kinase reaction, 2 mM of PNBM was added and the mixture was incubated overnight at 4 °C or during 2h at room temperature for alkylation. Laemmli 4x buffer (*tab.* 11) was then added to stop the reaction and the mixture was heated at 100 °C for several minutes before being concentrated by vivaspin concentrators (cut-off 10 kDa) and then loaded to 1.5 mm SDS-PAGE gel for western blotting.

After electro-blotting at 100V for 90 min. at 4°C, the blots were blocked with 5 % w/v milk in TBST (*tab.* 12) for 1 h. Primary antibodies against anti-thiophosphate ester were incubated with blocked membranes in the presence of 5 % w/v milk in TBST buffer for 1 h at room temperature at dilutions of 1:5,000. After three-times 10-min - washing in TBST, the membranes were probed with the second antibodies (anti-rabbit -HPR conjugate; Euromedex) for 1 h at room temperature at dilutions of 1:5,000. A new series of three-times 10-min washing in TBST was performed before the blot was revealed using Western exposure chemiluminescent detection system (Pierce). When indicated, the quantification of the signal correlating with incorporated phosphate was performed by ImageJ software.

## Phosphatase assay

*Phosphatase assay using pNPP*

p-Nitrophenyl phosphate (New England Biolabs) is a conventional non-proteinaceous substrate used to assay phosphatase activity based on the capacity of phosphatase to hydrolyze pNPP into p-Nitrophenol (pNP), a chromogenic product which gives absorbance at 405nm.

0.96  $\mu\text{M}$  of YdiC (25  $\mu\text{g}$ ) was added to 25 mM pNPP-containing cuvette of 1 mL in different phosphatase buffers. The release of pNP was continuously monitored by absorbance at 405 nm and it turns out that MES pH 5.0 is the optimal buffer (*tab.* 12). The specificity of pNPP phosphatase activity was confined to YdiC since no absorbance was detected in the absence of YdiC or in the presence of both YdiC and of phosphatase inhibitor cocktail 2 (Sigma). This inhibitor contains sodium orthovanadate, sodiul molybdate, sodium tartrate and imidazole.

*Phosphatase assay using radioactive ATP- $\gamma$ - $^{32}\text{P}$* 

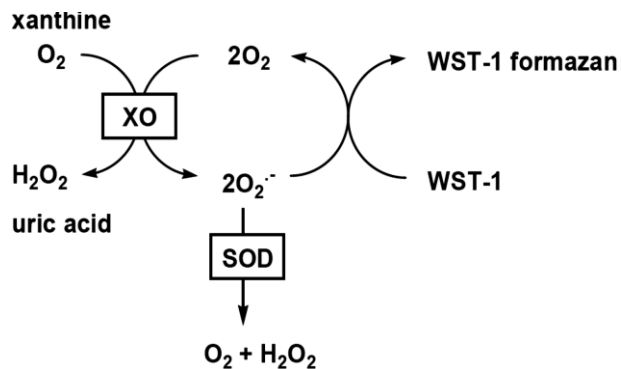
5  $\mu\text{M}$  of YdiC was added after 10 min. of incubation of 5  $\mu\text{Ci}$   $\gamma$ - $^{32}\text{P}$ -ATP with 5  $\mu\text{M}$  YdiB in the presence or absence of 5  $\mu\text{M}$  MBP at 30°C. In the control without YdiC, the same volume of buffer was added in place of YdiC. Samples were collected at 10, 30, 50, 80 and 110 min. after adding YdiC. Autoradiographic signals were quantified using ImageJ software.

*Phosphatase assay using ATP- $\gamma$ -S*

30  $\mu\text{M}$  of YdiC was added after 10 min. of incubation of 500  $\mu\text{M}$  ATP- $\gamma$ -S with 30  $\mu\text{M}$  YdiB in the presence of 15  $\mu\text{M}$  YdiE at 30°C. In the control without YdiC, the same volume of buffer was added in place of YdiC. Samples were collected at 0, 10, 20, and 50 min. after adding YdiC and submitted to overnight alkylation by 2 mM PNBM. After western blotting using anti-thio-phospho ester as the 1<sup>st</sup> antibody, blotted membrane was colored with Ponceau Red stain and signals were quantified using ImageJ.

## Superoxide dismutase assay

The enzyme superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion ( $\text{O}_2^{\cdot-}$ ) into hydrogen peroxide and molecular oxygen. In order to determine the SOD activity, an indirect method using Dojindo's highly water-soluble tetrazolium salt, WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD (*fig.* 55). Therefore, the inhibition activity of SOD as well as the  $\text{IC}_{50}$  (50% inhibition activity of SOD) can be determined by a colorimetric method.



XO and SOD antagonism in the generation of WST-1 formazan:

XO generates superoxide radical resulting in the reduction of WST-1 by superoxide anion to a colored WST-1 formazan product that absorbs light at 450 nm. SOD scavenges superoxide anion thereby antagonizes XO, reducing the rate of WST-1 formazan generation. Since the absorbance at 450 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 405 nm.

**Figure 55:** Principle of SOD activity assay

SodA (25  $\mu\text{M}$  final) was incubated with ATP (500  $\mu\text{M}$ ) in the presence or absence of YdiB (13  $\mu\text{M}$ ) at 30 °C overnight. 80 ng of SodA (phosphorylated by YdiB or non phosphorylated issued from the overnight reactions) was injected to SOD assay system in a quartz cuvette of 1 mL containing 400  $\mu\text{L}$  WST-1 and 40  $\mu\text{L}$  XOD (issued from the SOD assay kit from Sigma). Dilution buffer (included in the kit) was used in place of SodA for the blank. Formation of WST-1 formazan was followed by absorbance at 450 nm.

When indicated, the same protocol was used with SodA phosphorylated by bovine PKA. In this case, the overnight reactions contain ATP (1 mM), SodA (25  $\mu\text{M}$ ), activator cyclic-AMP (600  $\mu\text{M}$ ) in presence or absence of PKA (135  $\mu\text{g}$ , ~75 units).

To determine the  $\text{IC}_{50}$  of phosphorylated and non-phosphorylated SodA, different concentrations of each form of SodA were used.  $\text{IC}_{50}$  was defined as quantity of SodA necessary to inhibit 50% of WST-1 formation.

## Phosphoamino acid analysis

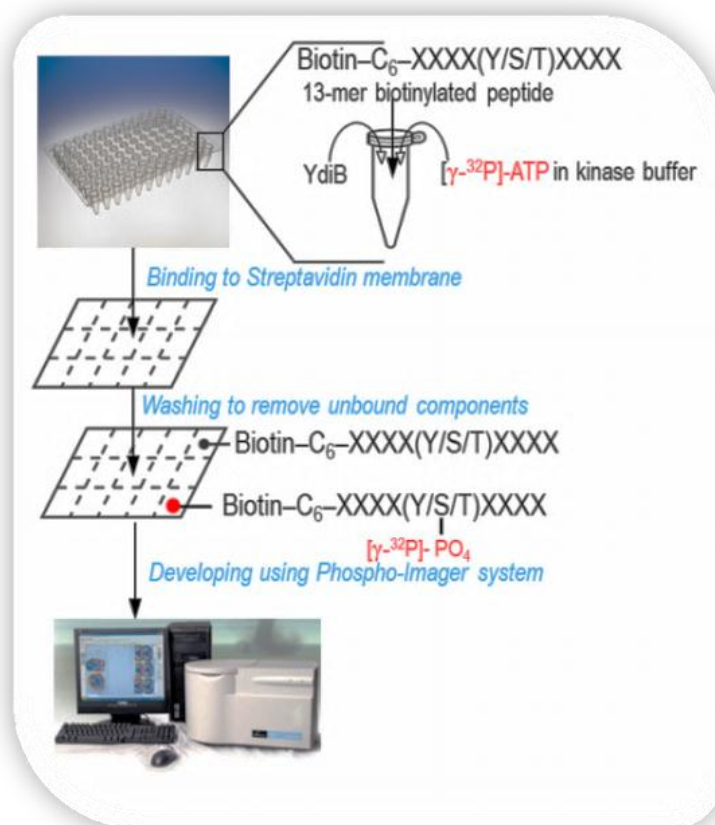
In order to identify the nature of phosphoamino acids which have been formed by covalent binding of phosphoryl groups in YdiB and its potential substrates, the following protocol developed by Grangeasse & al.<sup>798</sup> was used:

Kinase assays using radioactive ATP- $\gamma$ -<sup>32</sup>P were performed as previously described during 30 min at 30 °C. The mixture containing phosphorylated proteins were loaded onto 16 % acrylamide SDS-PAGE and the gels were run at 200V during 30 min. before being transferred onto PVDF membrane at 40 mA per membrane for 1 h. The labeled blotted protein-containing membranes were then stained with Ponceau Red and sliced protein-containing membrane pieces were washed with 600  $\mu$ L of methanol 100% then with 600  $\mu$ L of water and finally were incubated with 300  $\mu$ L of HCl 6N during 1 h at 110 °C in glass tubes then allowed to drain overnight in Speed-Vac concentrators.

After re-suspending the dried acid hydrolysates in 20  $\mu$ L of electrophoresis buffer, 5  $\mu$ L of standard phosphor-Ser, -Thr and -Tyr (Sigma) were added and 2  $\mu$ L of the mixture were subjected to two-dimensional thin layer chromatography (10  $\times$  10 cm; 0.1-mm layer thickness; Merck Inc.) in TLC solvent 1 for 90 min. and TLC solvent 2 for 30 min. at room temperature (*tab. 12*).

After migration and drying, the position of phosphoamino acid markers was visualized by TLC stain (*tab. 12*) and the plates were then exposed to X-ray films to locate the position of <sup>32</sup>P-labelled amino acids.

## In vitro peptide phosphorylation (Biotide assay)



**Figure 56:** Biotides assay schema

The membrane was extensively washed as recommended by the supplier to remove excess unbound radiolabeled ATP. The dried labeled-peptide containing-membrane was exposed to a phosphor screen and incorporated <sup>32</sup>P signal was measured using both PhosphoImager and Gel-Pro Analyser software. Phosphorylation score is expressed as a ratio of digital light units of <sup>32</sup>P incorporated into each peptide to digital light units of background obtained from a mean of the biotides with lowest intensity.

In order to get more insights into phosphor-sites of autophosphorylating YdiB as well as of its potential substrates, different biotides (# 112 - 170, *tab. 13*) containing phosphorylatable residues of YdiB, MBP, YdiD and EngA-GD2KH domain were also synthesized and biotide assays were performed as previously described.



**Table 13: Biotides used in this study**

#	Name	Sequence	#	Name	Sequence	#	Name	Sequence
1	AhpF	DELASMSKISVE	60	RsbRA	GVPVVDTMVAHHI	112	YdiB	KQLKWRVTVNPEET
2	AhpF	VLQKRLYSLPNVT	61	RsbRA	HHIIQASEAVRLV	113	YdiB	TVNPEETKAIKAKL
3	AroA	MSNTELELLRQKA	62	RsbS	SVDMIDSFIAKVL	114	YdiB	KAIKAKLTAFAKP
4	Asd	IVIDNTSAFRMDE	63	RsbV	ICLKDVSYMDSTG	115	YdiB	KPGDVLTLGDLG
5	Asd	EPIRKAYGLNKVI	64	RsbV	VSYMDSTGLGVFV	116	YdiB	LGAGKTTFTKGF
6	Mdh	ERVIGQSGVLDTA	65	Soda	VPENIRTAVRNGG	117	YdiB	AEGLGITRIVNSP
7	CitZ	TNLTGESKQWYEMS	66	SodaA	HTKHHNTYVTNLN	118	YdiB	TRIVNSPTFTIHK
8	CodY	RVGITRSVIVNAL	67	SpoIIAA	DLSFMDSSGLGVI	119	YdiB	FTIIEKYNDGVLP
9	CysS	NEKMSKSLGNFVL	68	SpoVG	THPINSSTRGKIQ	120	YdiB	GVLPYHMDVYRM
10	DegS	HARNRLSEVSRNF	69	SrfAA	FETGGHSLKAMTL	121	YdiB	LYHMDVYRMEDES
11	DhbC	EVPEKPSLIKTET	70	SrfAB	FMIGGHSKAMMM	122	YdiB	YRMEDESEDLGLD
12	DhbF	FELGGHSLLAARL	71	SrfAB	FDLGGHSLKGMML	123	YdiB	DLGLDEYFHGQGV
13	Drm	NGKDTMTGHWEIM	72	SrfAC	FALGGHSLKAMTA	124	YdiB	DEREITFTAVGNR
14	Eno	GGFNKSLTPVPM	73	SucC	AKLNFDNANLYRQ	125	YdiB	TAVGNRYEMLCEE
15	Eno	DGKYHLSGEGVVK	74	SucD	VQGITGSTALFHT	126	YdiB	LCEELSRHDNISN
16	Eno	TNTKKLSEGIKNG	75	TagE	MSLHAVSESNIKQ	127	YdiB	VNSPTFTIKEYN
17	Eno	SKYPIISIEDGLD	76	Tpi	RIQYGGSVKPAKI	128	YdiD	MKTKAAVRNMRLE
18	Eno	EELVSKYPIISIE	77	Tsf	VLTKDDSSAFGAY	129	YdiD	VYEIASSFTSPW
19	FbaA	LVLHGGTGIPTAD	78	YbbI	MSEPLNLHRLTTE	130	YdiD	SPWTKDSFYHELL
20	FbaA	AKINVTENQISS	79	YbbT	GVMISASHNPVQD	131	YdiD	ELLENPYAHYLV
21	Fus	AKLFDGSHYDVDS	80	YcnE	FLSEASLQVHRSR	132	YdiD	DGHLAGYCGIWIW
22	Fus	AEELRNSLIEAVC	81	Year	MDDLQFSMPMGVK	133	YdiD	MDDAQITNIAIKP
23	Fus	IHKIGETHEGASQ	82	YerB	QSQMPETVGPVRS	134	YdiD	AIKPEYRQSQLGE
24	Fus	ETHEGASQMDWME	83	YerB	TVGPVRSAREYFV	135	YdiD	SLGETLFRSAVEL
25	Fus	YEEVPKSAVEEII	84	YfiY	NLKAVKSGNAHEV	136	YdiD	KDARRLSLEVRVS
26	Fus	IDAGKTTTTTERIL	85	YfiY	VDDVWWTAGGIK	137	YdiD	HQAQGLYKKGFMQ
27	Fus	EIERHSSDEEPFS	86	YfkK	MKKESFSPSEMQA	138	YdiD	GIRKNYYTDNGED
28	Gap	ANHDVINSASCTT	87	YfkK	KESFSPSEMQAIA	139	YdiD	GEDALIMWVTINE
29	Gap	SNASCTTNCLAPP	88	YfnI	QTGQGKTSDAELT	140	MBP	MSNKFLGTWKLVS
30	GlpP	YRKTHKSLSLGHLK	89	YfnI	TSDAELTMDNSIF	141	MBP	GTWKLVSSENFDE
31	Hbs	MNKTELINAVAEA	90	YjdD	KRKFEASQREMGK	142	MBP	SENFDEYMKALGV
32	InfA	TVELSPYDLTRGR	91	YjoA	MGHTELPFYQORM	143	MBP	LGVGLATRKLGNL
33	RsbRB	LVGDIDTERAKFI	92	YloP	QEDEEMTKAIPII	144	MBP	KPRVVISKKGDI
34	Idh	DVKNAAYHIIIEKK	93	YnfE	KQYMVLYKKMSNM	145	MBP	DIITIRTESPFKN
35	LicB	YTIWAVSGDSVQN	94	YojH	LIGDIDTVRAKLI	146	MBP	ESPFKNTEISFKL
36	MtiA	KSEVLHSGISIIQ	95	rsbRC	DMITELSAPIVL	147	MBP	GQFEFETTADNRK
37	Ndk	GSDSLESAEREIN	96	YojH	VIVLFHVSGLLPL	148	MBP	RKTKSTVTLARGS
38	Ndk	KNIIHGSDSLESA	97	YorK	ILQLDGYKDYWGY	149	MBP	VTLARGSLNQQVK
39	Ndk	RQLIGKTNPKAEL	98	YpfD	VKVKLVSVDRDNE	150	MBP	KWDGNETTIKRKL
40	Ndk	IIHGSDSLESAER	99	YpoC	QELREKSYPAKPI	151	MBP	MKDQVCTRIKERV
41	OppA	NGGNNDTGWENPE	100	YpsB	KKQPVSNTTNFD	152	EngA-GD2KH	KYNEEVIQFLIG
42	OppA	EPIAGVYWKFNNT	101	YqbO	KSVKSSISETLFS	153	EngA-GD2KH	PNVGKSSLVNAML
43	OppA	IAGVYWKFNTEA	102	YqfN	EKSGLSHISVRQ	154	EngA-GD2KH	VIVSNVAGTTRDA
44	PdhB	DTVFPFQAESVW	103	YqhA	LVGEIDTHRARTI	155	EngA-GD2KH	DAVDTSFTYNQQE
45	PdhB	PFSQAESVWLPNH	104	YqjI	MVKAGTATDITI	156	EngA-GD2KH	EFVIVDTAGMRKK
46	Pgi	HNIHEKTGAGSDF	105	YtnP	VPKPLWSKKYPVN	157	EngA-GD2KH	KVYETTEKYSVLR
47	Pgk	VLGKAVSNPDRPF	106	YtxJ	KWHTSHSQITEAA	158	EngA-GD2KH	LKAIDRSEVVAVV
48	Pgk	IDIGTKTRETAYD	107	YvyG	LTKEQKYIQAITQ	159	EngA-GD2KH	DKRIAGYAHEAGK
49	Pgm	EGQMGNSEVGHNL	108	Ywfi	KLLSSDERQSIIH	160	EngA-GD2KH	AVDKDESTMKFEF
50	PnhA	VTVFGESAGGMSI	109	YwjH	AKEANVSFHDLRL	161	EngA-GD2KH	HFQFLDYAPILFM
51	Pnp	IGLILGSLGILA	110	Udg	RLNFETSYEKGLA	162	EngA-GD2KH	PILFMSALTKKRI
52	Pta	VSGAAHSTADTVR	111	YxxG	IFDDYKESKSNNSN	163	EngA-GD2KH	TKKRIHTLMPAII
53	PtsH	FKVTADSGIHARP				164	EngA-GD2KH	IIKASENHSRLRVQ
54	PtsH	KTVNLKSIMGVMS				165	EngA-GD2KH	HSLRVQTNVLNDV
55	PtsI	VEKKNISDSEAEV				166	EngA-GD2KH	MNPTPTHTNGSRLK
56	PykA	VARLNFSHGDFEE				167	EngA-GD2KH	RLKIYYATQVSVK
57	PyrB	NVKRGEAAYVISH				168	EngA-GD2KH	QVSVKPPSFVVV
58	RocA	MTVTYAHEPFTDF				169	EngA-GD2KH	PELMHFSYERFLE
59	RsbRA	LVGTIDTERAKRI				170	EngA-GD2KH	AFGFEGTPIKIFA

**Bioinformatic methods**

## Protein alignment and Phylogenetic analysis

*Data collection*

For **Histidine kinases** (fig. 5, I.1.3.3): 18 bacterial sensor histidine kinases from different two-component systems were collected from <http://www.ncbi.nlm.nih.gov/> with the following protein codes: PhoM-NP\_418816.1, PhoQ-NP\_460200.1, CheA,s-AEF07797.1, CheA,t-AAA96387.1, BvgS-AAA22970.1, DivL-AF083422\_1, CpxA-AAN83291.1, EnvZ-AP\_004386.1, PhoR-AAN78987.1, PgtP-CBW18467.1, DctB-CAA77619.1, SpoIIJ-AAA22800.1, NRII-ABR79556.1, DegS-NP\_391430.1, SpoIIAB-BAA12654.1, RsbW-NP\_388353.1, UhpB-YP\_859272.1, RsbT-NP\_388350.1

For **IDHK/P AceK** (fig. 8, I.1.3.5): *E. coli* AceK and 17 eukaryotic Ser/Thr protein kinases were collected from <http://www.ncbi.nlm.nih.gov/> with the following protein codes: PKC\_P04409, CDK2\_P24941, PKA\_P05132, INSR\_P06213, FGFR1\_P11362, SRC\_P32577, FRK\_P42685, 7LESS\_P13368, CLK1\_P49759, CDK1\_P00546, PhK\_P00518, PYK1\_P18160, DUN1\_P39009, CSK21\_P15790, MAPK2\_P49137, WEE1\_P30291, IME2\_P32581 and AceK\_P11071

For **HPrK/P** (fig. 12, I.2.2): HPrK/P from 9 Gram (+) and 3 Gram (-) bacteria were collected from <http://www.ncbi.nlm.nih.gov/> with the following protein codes: 20150941\_S.xyloso, 209540246\_S.pyogenes, 257158098\_E.faecalis, 23200250\_L.casei, 290580775\_S.mutans, 339292372\_S.salivarius, 3122218\_B.subtilis, 166079087\_M.genitalium, 2496289\_M.pneumoniae, 59800761\_N.gonorrhoeae, 319410106\_N.meningitidis, 71900936\_X.fastidiosa

For **BY kinases** (fig. 16, I.2.3): BY kinases from 5 Gram (+) and 7 Gram (-) bacteria were collected from <http://www.ncbi.nlm.nih.gov/> with the following protein codes: CapD\_S. thermophilus\_O87931, CpsD\_S. pneumoniae\_Q9AHD2, CpsD\_S. agalactiae\_Q3K0T, CapB\_S. aureus\_P39851, YwqD\_B. subtilis\_P96716, EspB\_R. solanacearum\_Q45409, AmsA\_E. pyrifoliae\_D0FRA8, AmsA\_E. amylovora\_E5B5N3, Wzc\_A. lwoffii\_Q9RMD9, Etk\_E. coli\_P38134, Wzc\_K. pneumonia\_A6TBF7, Ptk\_A. johnsonii\_O52788

For **Ubiquitous bacterial kinases Ubk** (fig. 21, II.1.1): 44 COG0802 proteins were collected from COG database <http://www.ncbi.nlm.nih.gov/COG/> with the following protein codes: Thermotoga\_TM1632, Synechocystis\_sll0257, Fusobacterium\_FN0929, Mycobacterium\_Rv3422c, Mycobacterium\_ML0377, Corynebacterium\_Cgl0589, Mycobacterium\_MT3531, Deinococcus\_DR2351, Chlamydia\_CT537, Chlamydomydia\_CPn0657, Bacillus\_ydiB, Bacillus\_BH0545, Streptococcus\_SP1944, Listeria\_lin2184, Lactococcus\_L44542, Streptococcus\_SPy1735, Escherichia\_O157\_H7\_ZyjeE, Escherichia\_K12\_yjeE, Pseudomonas\_PA4948, Vibrio\_VC0343, Haemophilus\_HI0065, Pasteurella\_PM0902, Mesorhizobium\_mli5086\_1, Caulobacter\_CC3534, Neisseria\_NMB0457, Neisseria\_NMA2027, Aquifex\_aq\_843, Xylella\_XF0758, Treponema\_TP0875, Borrelia\_BB0186, Campylobacter\_Cj0668, Helicobacter\_jhp0654, Helicobacter\_pylori\_HP0716, Rickettsia\_RP013, Escherichia\_EC5144, Yersinia\_YPO0369, Salmonella\_STM4357, Ralstonia\_RSc2540, Sinorhizobium\_SMc02757, Brucella\_BMEI2026, Rickettsia\_RC0013, Agrobacterium\_Agau\_C101044

For **revelation of new bacterial protein kinase branch** (fig. 25, I.1.4): 112 bacterial protein kinases were collected from Uniprot database <http://www.uniprot.org/> with the following protein codes: EnvZ\_P0AEJ4\_E.coli, RsbT\_P18392\_E.coli, CpxA\_P0AE84\_E.coli, PfeS\_Q04804\_Pseudomonas\_aeruginos, Atos\_Q06067\_E.coli, KinE\_O31661\_B.subtilis, raS\_P14377\_E.coli, Z, PhoR\_P23545\_B.subtilis, PiiS\_P33639\_Pseudomonas\_aeruginosa, SasA\_Q7V6P7\_Prochlorococcus\_marinus, HssS\_Q49ZT9\_Staphylococcus\_saprophyticus, YycG\_Q45614\_B.subtilis, PhoR\_P08400\_E.coli, PhoM\_P08401\_E.coli, PhoQ\_P14147\_Samonnella\_typhimurium, BarA\_Q11YX0\_Cytophaga\_hutchinsonii, PhoR1\_Q2S366\_Salinibacter\_ruber, MprB\_Q1B3X9\_Mycobacterium\_sp., PrrB\_P0A5Z8\_Mycobacterium\_tuberculosis, TrcS\_P96368\_Mycobacterium\_tuberculosis, CssS\_O32193\_B.subtilis, KinA\_P16497\_B.subtilis, Q9RE09\_Lactobacillus\_casei, O34483\_B.subtilis, Q9ZA98\_Strep-tococcus\_salivarius, Q9S1H5\_Staphylococcus\_xyloso, O07664\_Enterococcus\_faecalis, Q9ZA56\_Streptococ-cus\_mutans, P68898\_Streptococcus\_pyogenes, P47331\_Mycoplasma\_genitalium, P75548\_Mycoplasma\_pneu-monia, B4RK02\_Neisseria\_gonorrhoeae, A9M392\_Neisseria\_meningitides, Q9PDH3\_Xylella\_fastidiosa, P38134\_Etk\_E.coli, O52788\_Ptk\_Acinetobacter\_johnsonii, Q9RMD9\_Wzc\_Acinetobacter\_lwoffii, Q46631\_AmsA\_Erwinia\_amylovora, D0FRA8\_AmsA\_Erwinia\_billingiae, A6TBF7\_Wzc\_Klebsiella\_pneumoniae, Q45409\_EspB\_Ralstonia\_solanacearum, O32271\_YwqD\_B.subtilis, P39851\_CapB\_Staphylococcus\_aureus, O87931\_EpsD\_Streptococcus\_thermophilus, Q3K0T0\_CpsD\_Streptococcus\_agalactiae, Q9AHD2\_CpsD\_Streptococ-cus\_pneumoniae, P11071\_E.coli, B2TWJ8\_Shigella\_boydii, COQ489\_Samonnella\_paratyphi, A8ANB7\_Citro-bacter\_koseri, A4W5D0\_Enterobacter\_sp., A6TGR8\_Klebsiella\_pneumonia, C6DFQ3\_Pectobacterium\_caroto-vorum, A4TH34\_Yersinia\_pestis, A8GKF5\_Serratia\_proteamaculans, Q6D012\_Erwinia\_carotovora, Q7MZA0\_Photorhabdus\_luminescens, A0KMM4\_Aeromonas\_hydrophila, Q9I3W8\_Pseudomonas\_aeruginosa, B2JKI4\_Burkholderia\_phymatum, Q5P801\_Aromatoleum\_aromaticum, A1K4K7\_Azoarcus\_sp., Q487A4\_Colwellia\_psychrerythraea, Q47BD0\_Dechloromonas\_aromatic, Q3IHD9\_Pseudoalteromonas\_haloplanktis, B2AG85\_Cupriavidus\_taiwanensis, Mycobacterium\_tuberculosis (PknA\_P65726, PknB\_P0A5S4, PknD\_O05871, PknE\_P72001, PknF\_P72003, PknH\_Q11053, PknI\_P65730, PknJ\_P65732, PknL\_O53510), Myxococcus\_xanthus (MasK\_Q1DB00, Pkn1\_P33973, Pkn2\_P54736, Pkn3\_Q9XBQ0, Pkn4\_Q9S478, Pkn6\_P54738, Pkn7\_Q9XBP9, Pkn9\_Q1DEA2, Pkn10\_Q9S476, Pkn11\_Q9XBP8), RC0013\_Q92JQ4\_Rickettsia\_conorii, RP013\_Q9ZED0\_Rickettsia\_prowazekii, HI0065\_P44492\_Hamophilus\_influenzae, ALR2300\_O52749\_Nostoc\_sp., SLL0257\_P74415\_Synechocystis\_sp., Rv3422c\_P67171\_Mycobacterium\_tuberculosis, ML0377\_Q49864\_Mycobacterium\_leprae, SCO4747\_O86788\_Streptomyces\_coelicolor, AQ\_843\_O67011\_Aquifex\_aeolicus, YdiB\_O05515\_B.subtilis, YjeE\_P0AF68\_E.coli, Spr1761\_Q8DNF8\_Streptococcus\_pneumoniae.

*Protein sequence multiple-alignment and phylogenetic tree analysis*

Protein sequence multiple-alignments were performed with the program ClustalX<sup>479</sup> using Neighbour Joining (NJ) method with 10000 bootstrap trials. The results were submitted to ESPript<sup>800</sup> server for the final visualization of aligned sequences.

Phylogenetic trees were constructed with Treeview<sup>470</sup> and/or Jalview<sup>444</sup> based on the aligned sequences previously obtained with ClustalX. When revealing the new branch of bacterial protein kinases, the aligned sequences were submitted to iTOL<sup>471,472</sup> server for visualization.

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## Modelization

### *Modelization of monomeric YdiB and its protein mutants of conserved residues*

Based on the C chain of *Haemophilus influenzae* YjeE (1htw.pdb)<sup>8</sup>, the model apo-structure of *B. subtilis* YdiB was constructed by 3 different methods: Modeller software<sup>473</sup>, and servers of ESYPred3D<sup>474</sup> and Swiss3D<sup>475</sup>.

The model of nucleotide-binding site of YdiB was constructed by superposing ADP-bound YjeE and Modeller apo-structure of YdiB using Pymol 1.3 software.

Different protein mutants of YdiB were modelized using the "Mutagenesis" function in Pymol. When needed, a molecule of ATP was introduced in place of ADP by using the "Pair Fitting" function in Pymol.

### *Modelization of MBP-monomeric YdiB complex*

The model of apo-YdiB (previously described) and the model of MBP (1qcl.pdb) were submitted to Pdb2pqr 1.7.1 server<sup>512</sup> for Poisson–Boltzmann electrostatics calculations and the resulting electrostatic potential maps were visualized via Pymol 1.3 software.

By using Pathdoc beta 1.3 server<sup>513,514</sup>, several candidate-complexes kinase-substrate YdiB-MBP were constructed. The best docking solution in term of Geometric shape complementarity score was chosen for calculating electrostatic potentials as described previously.

### *Modelization of tetrameric YdiB and YdiE*

The C chain of YjeE and the model of monomeric YdiB were submitted to the Symmdock beta 1.0 server<sup>513,542</sup>. Several candidate-homotetramers of YjeE or YdiB were established. The best docking solution in term of Geometric shape complementarity score was chosen for calculating electrostatic potentials via Pdb2pqr 1.7.1 server<sup>512</sup>. Pymol 1.3 software was used for visualization and analysis of oligomerization involving conserved residues. Finding of residues located in inter-subunit interfaces were achieved with help of InterProSurf server<sup>543</sup>.

## **Cell biology methods**

### Sub-cellular localization using immunofluorescence microscopy

*B. subtilis* strain mutant 1049 contains the gene *rspB* (encoding ribosomal protein S2) fused to *gfp* and both genes are under P<sub>XYL</sub> control (*tab.* 10). 200  $\mu$ L overnight pre-culture were added into 20 mL LB containing 1 % xylose (w/v). Once expressed, GFP has maximum absorbance at 396 nm and maximum emission at 508 nm with green fluorescence. Culture was grown to an OD<sub>600</sub> of  $\sim$ 0.6 then diluted into OD<sub>600</sub>  $\sim$  0.1. At this step, tetracycline or rifampicin were added when indicated to a final concentration of 3  $\mu$ g/mL or 0.05  $\mu$ g/mL respectively. After 2 more hours at 30 °C, cells were harvested and fixed overnight at 4 °C in fixing solution (*tab.* 12).

Fixed cells were washed three times in PBS before addition of lysozyme to a final concentration of 2 mg/mL for 2 min. Permeabilized cells were then transferred onto poly-L-lysine-coated Poly Prep slides (Sigma) and the slides were washed twice with PBS, air-dried, dipped in methanol at  $-20$  °C for 10 min and allowed to dry again. After rehydration with PBS, the slides were blocked for 1 h at room temperature in saturation buffer (*tab.* 12) then for 1 h with anti-YdiB antibodies (from Cocalico Biologicals) diluted at 1:50 in saturation buffer. The slides were then washed for 10 min in PBS and incubated with a 1:300 dilution of Cy3-conjugated goat anti-rabbit IgG (from Jackson Immunoresearch) in saturation buffer. CY3 has maximum absorbance at 554 nm and maximum emission at 568 nm with orange fluorescence.

For DNA staining, the slides were incubated for 15 min with 2 mg/mL DAPI (Euromedex) which forms fluorescent complexes with natural double-stranded DNA to have maximum absorption at 358 nm and maximum emission at 461 nm with blue fluorescence. After extensive washing with PBS, the slides were mounted using Mowiol solution (Sigma-Fluka).

Samples were observed and photographed with an Olympus fluorescence microscope equipped with a 6100 immersion objective.

### Growth under oxidative stress condition

#### *Disk inhibition assay*

*B. subtilis* WT-spec<sup>R</sup> and  $\Delta$  *ydiB*-spec<sup>R</sup> strains (*tab.* 10) were grown at 37°C overnight in LB medium, diluted 1:100 into fresh LB medium, and grown at 37°C with shaking. Mid-log phase WT and  $\Delta$ ydiB cultures were harvested and the pellets were then re-suspended into fresh LB medium with 50  $\mu$ g/mL of spectinomycin. 1 mL of fresh WT and  $\Delta$ ydiB cultures (OD<sub>600nm</sub> = 0.14 and 0.148 respectively) were separately mixed with 50 mL of agar LB (at  $\sim$  40°C) and poured evenly onto sterile petri dishes. After cooling, filter paper disks (7-mm diameter) carrying chemicals to be tested (100 ng of paraquat, norfloxacin or tetracycline) were placed onto the centre of the agar plate. The diameters of the inhibition zone were measured after the plates were incubated at 37°C for 24 hours. Experiments were repeated twice.

*Gradient plate assay*

*B. subtilis* WT-spec<sup>R</sup> and  $\Delta ydiB$ -spec<sup>R</sup> strains (*tab.* 10) were grown in LB medium at 37°C overnight, diluted 1:100 into fresh LB medium, and grown at 30°C with shaking. Dilutions ( $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$ ) of mid-log phase WT and  $\Delta ydiB$  cultures (OD<sub>600nm</sub> = 0.43 and 0.48 respectively) were spotted onto LB agar plates containing different concentration of paraquat (0-100  $\mu$ M) and incubated at 37°C overnight. This experiment was repeated twice and similar results were observed.

*Growth curves in liquid media*

One clone of each *B. subtilis* WT-spec<sup>R</sup> and  $\Delta ydiB$ -spec<sup>R</sup> strains (*tab.* 10) was individually re-suspended into 1 mL of LB containing 50  $\mu$ g/mL spectinomycin. 100  $\mu$ L of these bacterial cultures were individually added into 50 mL of LB media containing different paraquat concentration (0-100  $\mu$ M), and grown at 30°C with shaking. Growth curves of WT and  $\Delta ydiB$  were followed by absorbance at 600 nm during 36 hours.



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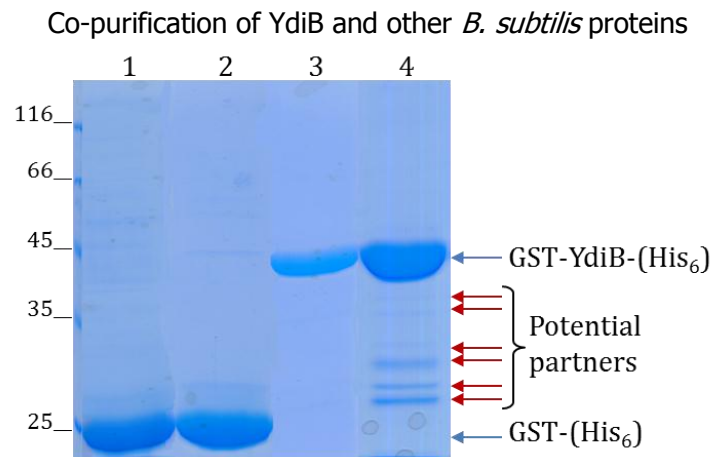
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*Supplementary  
data*

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## SUPPLEMENTARY DATA



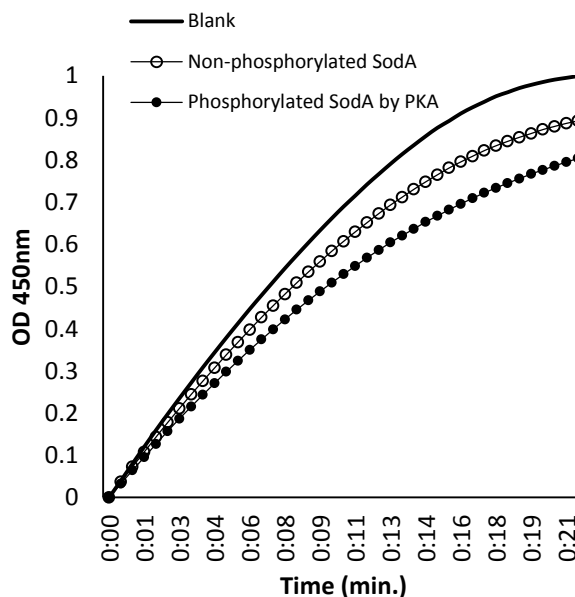
**Figure S1:** Co-purification of GST-YdiB-(His)<sub>6</sub> with other *B. subtilis* revealed by Pull-down method  
Purified GST-YdiB-(His)<sub>6</sub> and the control GST-(His)<sub>6</sub>, as the bait proteins, were incubated alone (lane 3 and 1 respectively) or with immobilized glutathione resin. Captured bait proteins were then incubated with *B. subtilis* lysate containing putative prey proteins. After washing to remove unbound proteins, retained components were eluted and separated by SDS-PAGE. Several additional bands were revealed in case of GST-YdiB-(His)<sub>6</sub> (lane 4) but not with the control GST-(His)<sub>6</sub> (lane 2). These bands containing potential partners of YdiB were then identified by mass spectrometry and presented in the (tab. x)

**Table S1:** Potential partners of YdiB revealed by Pull-down coupled with Mass spectrometry

Potential partners	Function
<b>SodA</b> Superoxide dismutase	Detoxifies oxygen radicals via reaction: (2 superoxide + 2 H <sup>+</sup> → O <sub>2</sub> + H <sub>2</sub> O <sub>2</sub> ), and thus contributes to resistance against oxidative stress <sup>653</sup> . <i>In vivo</i> phosphorylated at Thr34 and Thr70 in <i>B. subtilis</i> <sup>28</sup>
<b>AhpC</b> , small subunit of alkyl hydroperoxide reductase	Reduces reactive oxygen species (ROS) thus contributes to resistance against peroxide stress <sup>657</sup> ; is induced under conditions of Fe or Mn <sup>2+</sup> limitation <sup>656</sup> . <i>In vivo</i> phosphorylated in <i>B. subtilis</i> <sup>88</sup>
<b>SufC</b> , ABC transporter protein	ATPase component of the ubiquitous SUF machinery (SufABCDSE) involving in the biosynthesis of Fe-S clusters <sup>801</sup> ; <i>In vivo</i> phosphorylated at Ser/Thr/Tyr in <i>B. subtilis</i> <sup>88</sup>
<b>YceD</b> , general stress protein	required for survival of ethanol stress; might be implicated in oxidative stress response <sup>802</sup>
<b>YvyD</b> , general stress membrane protein	Unknown function; phosphate starvation-inducible protein <sup>803</sup> ; belongs to S30Ae ribosomal proteins-σ <sup>54</sup> modulation protein family.
<b>L1</b> , RplA large subunit ribosomal protein	Binds to 23S-rRNA and interacts with the ribosome-associated GTPase EngB <sup>630</sup> ; Localizes near the ribosomal binding site of elongation factor EF-Tu. <i>In vivo</i> phosphorylated in <i>E. coli</i> <sup>584</sup> , <i>L. lactis</i> <sup>113</sup> and <i>Streptococcus pneumoniae</i> <sup>596</sup>
<b>S3</b> , RpsC small subunit ribosomal protein	Binds to mRNA and position it for the decoding; Interacts with S10 and S14 <i>In vivo</i> phosphorylated in <i>E. coli</i> <sup>584</sup> , <i>Streptomyces collinus</i> <sup>591</sup> , <i>Streptomyces coelicolor</i> <sup>593</sup> and <i>Listeria monocytogenes</i> <sup>597</sup>
<b>PyrH</b> Urydilate kinase	Pyrimidine biosynthesis via phosphorylation of UMP
<b>PyrE</b> Orotate phosphoribosyltransferase	Pyrimidine biosynthesis via formation of OMP (precursor of UMP) by transferring phospho-ribose group from phosphoribosyl-pyrophosphate onto orotate



## Up-regulation of SOD activity of SodA upon phosphorylation



**Figure S2:** Phosphorylation of *B. subtilis* SodA by bovine heart PKA increases SOD activity

Xanthine Oxidase (XOD) generates superoxide radical resulting in the reduction of WST-1 by superoxide anion to a colored WST-1 formazan product that absorbs light at 450 nm. SOD scavenges superoxide anion thereby antagonizes XOD, reducing the rate of WST-1 formazan generation. SodA (60  $\mu$ g) was incubated with ATP (1 mM) in the presence or absence of PKA (135  $\mu$ g, ~75 units) at 30°C overnight. 80ng of SodA (phosphorylated --●-- or non-phosphorylated --○--) was injected to SOD assay system containing WST-1 and XOD. Dilution buffer (supplied in the kit) was used in place of SodA in the blank ( \_\_\_ ). Formation of WST-1 formazan was followed by absorbance at 450nm. SOD activity colorimetric assay kit was supplied by Fluka-Simga-Aldrich®.

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## Abstract :

Genome sequencing data has revealed genes encoding uncharacterized protein family UPF0079 which are exclusively found in bacteria; broadly distributed in this kingdom and possess an ATP-binding motif in their sequences. Biochemical characterization and physiological role elucidation of UPF0079 will undoubtedly increase our fundamental biology knowledge, and also remain a prerequisite towards the development of new antimicrobial compounds. Our investigation on YdiB, an archetype of this family in *Bacillus subtilis* revealed both autophosphorylating and protein phosphotransferase activities. The dual-specificity Ser/Thr and Tyr kinase activity of YdiB seems to require oligomerization is upregulated by basic molecule activators such as natural polyamines or poly-L-lysine. The 10 most conserved residues were studied to gain insights into molecular mechanism of the kinase YdiB. To characterize the function of phosphorylation events linked to YdiB, starting with the *B. subtilis ydiA-B-C-D-E* operon we showed that YdiB and YdiC function as cognate protein kinase/phosphatase towards two ribosome-related protein substrates YdiD and YdiE. Some co-localization between YdiB and ribosomes were observed. Furthermore, YdiB is capable of phosphorylating both the ribosomal 50S and 30S subunits as well as two ribosome-binding GTPases EngA and EngB. We also demonstrated that phosphorylated EngA by YdiB is an *in vitro* substrate of the phosphatase YdiC. Finally, based on the phosphoproteome of *Bacillus subtilis*, peptides mimicking the *in vivo* phosphorylation sites were used. Some of them were found to be phosphorylated *in vitro* by YdiB, including two peptides which belong to the superoxide dismutase SodA. The activity of purified SodA was then shown to be upregulated via phosphorylation by YdiB. We furthermore found that *B. subtilis* cells lacking *ydiB* become more sensitive to oxidative stress-causing agents such as paraquat or norfloxacin. We propose that *in vivo*, YdiB functions as a protein kinase involved in ribosome function in normal condition; and in protecting cells from oxidative stress damage.

Keywords : kinase, phosphatase, phosphorylation, YdiB, *Bacillus subtilis*, ribosome, oxidative stress

## Résumé :

Les données de séquençage des génomes ont révélé la famille UPF0079 comprenant des protéines de fonction inconnue qui sont exclusivement présentes chez les bactéries; largement distribuées dans ce règne et possèdent le motif A de Walker dans leur séquence. La caractérisation biochimique et l'élucidation du rôle physiologique de cette famille contribueront à élargir nos connaissances en biologie fondamentale, et sont également un préalable vers le développement de nouveaux composés antimicrobiens. Notre étude sur YdiB, un archétype de cette famille chez *Bacillus subtilis* a révélé à la fois l'autophosphorylation de YdiB et son activité de protéine kinase. L'activité kinase de double spécificité Ser/ Thr et Tyr de YdiB semble nécessiter son oligomérisation et semble être stimulée par des molécules basiques telles que des polyamines naturelles ou la poly-L-lysine. Les 10 résidus les plus conservés chez cette famille ont été étudiés afin de mieux comprendre le mécanisme moléculaire de YdiB. Concernant la caractérisation fonctionnelle de la phosphorylation liée à YdiB, l'étude de l'opéron *ydiA-B-C-D-E* de *B. subtilis* nous a permis de montrer que YdiB et YdiC fonctionnent comme un couple de protéine kinase/phosphatase de deux protéines substrats dont les fonctions seraient liées aux ribosomes, YdiD et YdiE. Une co-localisation partielle entre YdiB et les ribosomes a été observée. En outre, YdiB est capable de phosphoryler des protéines ribosomiques appartenant aux deux sous-unités 50S et 30S, ainsi que deux GTPases liées aux ribosomes, EngA et EngB. Nous avons également démontré que EngA phosphorylée par YdiB est un substrat *in vitro* de la phosphatase YdiC. Enfin, basé sur le phosphoprotéome de *Bacillus subtilis*, des peptides mimant des sites de phosphorylation *in vivo* ont été utilisés. Certains d'entre eux ont été trouvés à être phosphorylés *in vitro* par YdiB. Deux de ces peptides appartient à la superoxyde dismutase SodA dont l'activité *in vitro* et après purification est régulée positivement via la phosphorylation par YdiB. Nous avons ensuite constaté que les cellules de *B. subtilis* dépourvues du gène *ydiB* sont plus sensibles aux agents oxydants tels que le paraquat ou la norfloxacin. Nous proposons que, *in vivo*, YdiB fonctionne comme une protéine kinase impliquée dans la fonction des ribosomes dans des conditions physiologiques normales, et son activité kinase contribuerait à protéger les cellules contre les dommages du stress oxydatif.

Mots-clés : kinase, phosphatase, phosphorylation, YdiB, *Bacillus subtilis*, ribosome, stress oxydatif