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Bacterial Protein-Tyrosine Kinases

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Abstract: Bacteria and Eukarya share essentially the same family of protein-serine/threonine kinases, also known as the Hanks-type kinases. However, when it comes to protein-tyrosine phosphorylation, bacteria seem to have gone their own way. Bacterial protein-tyrosine kinases (BY-kinases) are bacterial enzymes that are unique in exploiting the ATP/GTP-binding Walker motif to catalyze phosphorylation of protein tyrosine residues. Characterized for the first time only a decade ago, BY-kinases have now come to the fore. Important regulatory roles have been linked with these enzymes, via their involvement in exopolysaccharide production, virulence, DNA metabolism, stress response and other key functions of the bacterial cell. BY-kinases act through autophosphorylation (mainly in exopolysaccharide production) and phosphorylation of other proteins, which have in most cases been shown to be activated by tyrosine phosphorylation. Protein-tyrosine phosphorylation in bacteria is particular with respect to very low occupancy of phosphorylation sites *in vivo*; this has represented a major challenge for detection techniques. Only the recent breakthroughs in gel-free high resolution mass spectrometry allowed the systematic detection of phosphorylated tyrosines by phosphoproteomics studies in bacteria. Other pioneering studies conducted in recent years, such as the first structures of BY-kinases and biochemical and physiological studies of new BY-kinase substrates significantly furthered our understanding of these enzymes and highlighted their importance in bacterial physiology. Having no orthologues in Eukarya, BY-kinases are receiving a growing attention from the biomedical field, since they represent a particularly promising target for anti-bacterial drug design.

Keywords: Protein-tyrosine kinase / BY-kinase / bacteria / protein phosphorylation / phosphoproteome / signal transduction / cellular regulation

BY-KINASES IN BACTERIAL PHYSIOLOGY

The literature in the research field of post-translational modifications of proteins abounds with statements that elaborate modifications found in *Eukarya* do not exist in *Bacteria*. On more than one occasion, this simply reflected the fact that nobody had made a serious effort looking for them. Recent examples of “missing” modifications finally found in bacteria are those of protein glycosylation [1], acetylation [2] and methylation [2]. In much the same spirit, protein-tyrosine phosphorylation was considered non-existent in bacteria, until the pioneering studies appeared in the mid nineties [3,4], identifying phosphorylated tyrosine residues in bacterial proteins. These first studies reported members of a new family of proteins that autophosphorylated on tyrosine, which were later named BY-kinases (for bacterial tyrosine kinases) [5], but are also known as polysaccharide copolymerases [6] due to multiple roles they play in the cellular physiology. BY-kinase-encoding genes have since been identified in a majority of sequenced bacterial genomes, and are thus considered widespread, if not ubiquitous, in the bacterial kingdom [7]. BY-kinases account for most tyrosine phosphorylation events identified in bacteria so far, the exception being some unusual two-component systems that phosphorylate on tyrosine. A typical BY-kinase

consists of an N-terminal transmembrane loop (of variable size), followed by a cytosolic catalytic domain that contains the ATP-binding site and a C-terminal tail of autophosphorylatable tyrosine residues [5]. A distinctive feature of these enzymes is that they use a structural motif known as the P-loop (Walker motifs A and B) to constitute their active site. This feature is shared by only a few bacterial protein-kinases [8], whereas in *Eukarya*, Walker motifs are found in many ATP/GTPases [9], but not in protein kinases. In *Firmicutes*, the canonic BY-kinase 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 separated to independent polypeptide chains (Fig. 1). The cause and functional consequences of this separation remain unclear, although some new leads have appeared in recent studies and these will be discussed in the following chapter.

In the first phase of research on BY-kinases, since their discovery in 1996 and up to 2003, these enzymes were considered exclusively as autophosphorylating kinases. Initially, BY-kinase autophosphorylation has been linked to the functional role of these enzymes in exopolysaccharide production in several bacterial systems that were studied, since they are usually encoded by genes in the large operons involved in biosynthesis and export of sugar polymers [10]. One of the most exhaustively studied systems concerning BY-kinase implication in exopolysaccharide synthesis is the *Proteobacteria* model organism *Escherichia coli*. *E. coli* possesses two BY-kinases: *Wzc* encoded a gene in the operon which participates in the biosynthesis of polysaccharide polymers [11]

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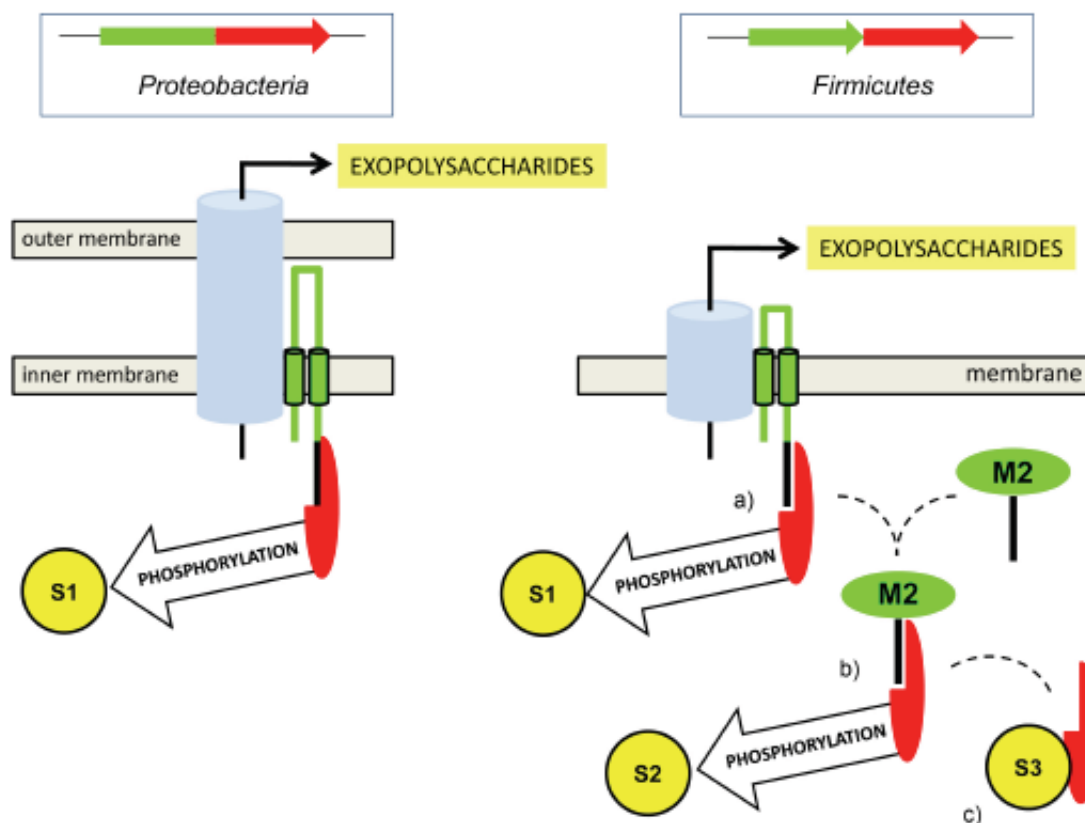


Fig. (1). Schematic representation of the two archetypal BY-kinase architectures. *Proteobacteria* have the two functional domains; transmembrane (green) and kinase (red) encoded by a single gene, and in *Firmicutes* these domains are independent proteins, encoded by separate genes. BY-kinases in both types of bacteria interact with the exopolysaccharide synthesis and translocation machinery (light blue), which is more complex in *Proteobacteria* where it has to traverse 2 membranes. The C-terminal end of the transmembrane domain that activates the kinase domain is represented in black. In *Proteobacteria*, this fragment is linked covalently to the kinase domain, and the active kinase domain is thus capable of phosphorylating the full set of its endogenous substrates (S1). In *Firmicutes*, the kinase is also activated by the C-terminal fragment of the transmembrane modulator, and can thus phosphorylate a set of physiological substrates (S1), as shown in case a). However, the kinase is free to dissociate from this modulator and meet other proteins that can accomplish this function (M2), and perhaps change its specificity towards another set of substrates (S2), as illustrated in case b). Finally, recent data indicate that the kinase can also co-localize with some of its substrates (S3), case c), although the physiological role of this co-localization is not clear at present.

and Etk, encoded by a gene in the G4C operon which is required for formation of group 4 capsule (G4C) polysaccharide [12]. It has been demonstrated that presence of Wzc and Etk is essential for synthesis of corresponding extracellular polysaccharides. More to the point, the auto-phosphorylation of tyrosine residues in the C-termini of these BY-kinases is the key feature in the assembly of capsular polysaccharides. Both the phosphorylated and the non-phosphorylated forms of Wzc are essential for polysaccharide polymer production [13,14], and the current hypothesis is that the cycling between phosphorylated and non-phosphorylated form of Wzc allows the polysaccharide polymer synthesis to proceed correctly. Accordingly, Wzc and Etk have been classified as polysaccharide co-polymerases. In their co-polymerase capacity, these BY-kinases have been shown to influence the amount of polysaccharides as well as the length of the produced polymer probably via an interaction with a polysaccharide polymerase Wzy [14-16]. An interesting observation from the evolutionary standpoint is that there are members of the polysaccharide co-polymerase family which contain the transmembrane domain, but have no cytosolic kinase domain

[6]. A similar system has been described in *Firmicutes*, where the most extensively studied system with respect to exopolysaccharide production is probably that of *Streptococcus pneumoniae*. The autophosphorylating BY-kinase CpsD is essential for capsular polysaccharide biosynthesis in *S. pneumoniae*, where it regulates the amount of the synthesized capsular polysaccharide, which in turn affects the attachment of the bacteria to the host cells and contributes to the infection process [17,18]. Similar processes have been described in a number of other bacteria [5]. An interesting hypothesis has been put forward by Cuthbertson *et al.* [6] who argue that co-polymerases may simply influence the amount of produced polysaccharides by serving as molecular scaffolds for the other members of the translocon. The detailed mechanism of the functional interaction between polymerases and co-polymerases has not been entirely pinpointed, but considerable efforts are currently directed at this question.

After the initial link between BY-kinase autophosphorylation and exopolysaccharide production had been firmly

established, this field took a new direction in the year 2003, when several teams simultaneously published reports that BY-kinases can phosphorylate other endogenous proteins substrates on tyrosine residues [19-21]. In each reported case the substrate activity was regulated by BY-kinase dependent phosphorylation (Fig. 2). *E. coli* Etk was found to phosphorylate RpoH (an alternative heat shock sigma factor) and an anti-sigma factor RseA and thus participate in triggering the heat shock response [19]. Its paralogue, Wzc [20], and a BY-kinase orthologue from *Bacillus subtilis*, PtkA [21], were found to phosphorylate UDP-glucose dehydrogenases in their respective organisms. The molecular mechanism of activation of UDP-glucose dehydrogenases by BY-kinase dependent tyrosine phosphorylation has been studied in molecular detail [22-24], and in the process, Etk from *E. coli* has also been found to phosphorylate the cognate UDP-glucose dehydrogenases [23]. Although the mechanisms of UDP-glucose dehydrogenases activation are arguably not identical in *E. coli* [23] and *B. subtilis* [24], they both involve precise positioning of the phosphorylated tyrosine with respect to the bound substrate in the enzyme active site. Moreover, the investigation of the activation mechanism in *E. coli* has shed light on two parallel signal transduction pathways that intersect at the level of UDP-glucose dehydrogenase phosphorylation; one involving Wzc and the colanic acid synthesis and the other Etk and polymyxin resistance [23]. Examples of BY-kinases phosphorylating enzymes involved in the synthesis of sugar polymers have rapidly emerged in other bacterial systems. *S. thermophilus* phosphoglycosyltransferase EpsE is phosphorylated and activated by the cognate BY-kinase EpsD [25], *Staphylococcus aureus* BY-kinase Cap5B2 can phosphorylate and activate an endogenous

UDP-acetyl-mannosamine dehydrogenase Cap5O [26], whereas tyrosine-phosphorylation of *Klebsiella pneumoniae* undecaprenolphosphate glycosyltransferase WcaJ has been reported as necessary for capsular polysaccharide synthesis [27]. The emergent phosphorylation networks based on BY-kinases and their substrates hold promise of significant complexity. So far only one case of a single substrate phosphorylated by several kinases has been reported: *E. coli* Ugd phosphorylated by Wzc and Etk [23]. However, the examples of one kinase phosphorylating several substrates are plenty: *E. coli* Etk phosphorylates RpoH, RseA [19] and Ugd [23], and *B. subtilis* PtkA phosphorylates UDP-glucose dehydrogenases Ugd and TuaD [21], as well as single-stranded DNA-binding proteins SsbA and SsbB [28]. Phosphorylation of single-stranded DNA-binding proteins by BY-kinases, which results in increased affinity for DNA, hinted at an interesting possibility that BY-kinases might in fact be extensively involved in regulating the DNA metabolism. *B. subtilis* cells devoid of PtkA exhibited a complex pleiotropic phenotype with defects in the cell cycle, initiation of the DNA replication and chromosome distribution [29]. In addition to SsbA and SsbB, PtkA phosphorylates and regulates the activity of other proteins involved in *B. subtilis* single-stranded DNA metabolism, which may account for the complex phenotype of the $\Delta ptkA$ strain (Jers & Mijakovic, unpublished results). In *E. coli*, the BY-kinase Wzc was found to phosphorylate integrase proteins (Int) of coliphages HK022 and λ . Overexpression of Wzc in tyrosine-phosphatase deficient background resulted in a significantly reduced lysogenization, indicating that phosphorylation of Int down-regulates its activity [30].

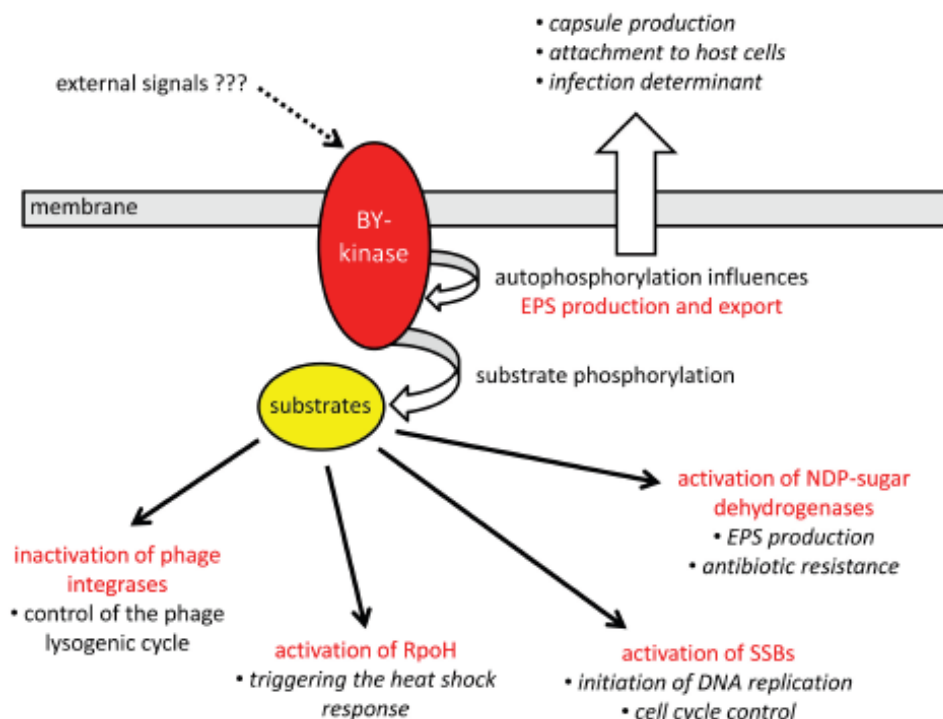


Fig. (2). Schematic representation of the physiological and regulatory roles of BY-kinases. BY-kinase autophosphorylation controls the involvement of these enzymes in the synthesis and translocation of sugar polymers. In addition, BY-kinases phosphorylate a number of intracellular substrates. Only well-studied cases are presented here, with clearly documented consequences for bacterial physiology.

Following the finding that heat shock response in *E. coli* is modulated by the activity of the BY-kinase Etk [19], a link between tyrosine phosphorylation and heat shock was also published for *B. subtilis*. A protein with no homology to canonical BY-kinases, McsB, was found to phosphorylate CtsR, repressor of the heat-shock genes in the presence of a modulator protein McsA [31]. By consequence, the tyrosine-phosphorylated CtsR was reported to release its target DNA. McsB was also found to act as a regulated adaptor protein for ClpCP, the protease complex responsible for the degradation of CtsR [32]. The active, phosphorylated kinase McsB interacts with both CtsR and ClpC, and targets CtsR to the ClpCP complex. Finally, in 2009 McsB seems to have been taken off the list of tyrosine kinases, and its status revised to that of an arginine kinase [33]. It would be interesting to see whether this kinase has a dual specificity, or the discrepancies between the two reports were due to different detection methods. This by no means diminished the importance of previous findings that McsB functionally interacts with CtsR and ClpCP. At the same time, this new finding opened a new chapter of bacterial protein phosphorylation at arginine residues.

PROTEOBACTERIA VS. FIRMICUTES: DIFFERENT STRUCTURES FOR DIFFERENT REGULATORY STRATEGIES?

Important advances in the field of bacterial protein-tyrosine kinases were made recently in terms of structural studies. The Walker motifs found in BY-kinases, and the overall sequence homology, had suggested that these proteins might structurally resemble bacterial ATPases such as MinD and Soj [5]. The first structural insights came from the low resolution structure (14 Å) of *E. coli* BY-kinase Wzc in complex with the capsular polysaccharide translocon Wza [34]. It revealed a Wzc tetramer that oligomerises via the periplasmic domains, and with its cytosolic kinase domains freely protruding into the cytosol (not interacting with each other). The functional insights derived from this structure suggested that Wzc regulates export by triggering an open/active conformation of the translocase Wza. More recently, two high resolution structures of BY-kinases were published: Etk from *E. coli* [35] representing the *Proteobacteria*-type BY-kinase architecture, and CapA/B from *S. aureus* [36] representing the *Firmicutes*-type BY-kinase architecture. These structures provided insights into the functional role of several conserved features. In a previous biochemical study it was demonstrated that Etk homologue Wzc is activated through a two-step mechanism involving a conserved tyrosine in the active site and the C-terminal tyrosine cluster [37]. The structure data combined with biochemical and *in silico* analysis revealed a novel mechanism by which the first activation steps proceeds, showing that the conserved tyrosine side chain points into the active site thereby blocking activity. Upon autophosphorylation, the negatively charged phosphotyrosine rotates out of the active site and is stabilized by interaction with an arginine residue [35,38]. In *Firmicutes*, where the kinase domain is separated in a distinct polypeptide chain, it has been shown that the interaction with the remaining transmembrane domain is necessary for the kinase activity [21,39]. The structure of *S. aureus* CapB demonstrated that the activation proceeds, at least in part, by

the fact that the C-terminal part of the transmembrane modulator (protruding into the cytosol) stabilizes ATP binding by hydrophobic sandwich interaction between the adenine ring and a phenylalanine residue of the modulator protein [36]. In case of CapB, the non-phosphorylated kinase monomers, as opposed to the case of Wzc, associated in an octameric ring structure, anchored to the membrane via interaction with the transmembrane modulator CapA. Notably, one of the tyrosine residues in the C-terminal tyrosine cluster was found bound to the active site of the neighbouring subunit, suggesting an inter-molecular phosphorylation mechanism. The fact that no tyrosine residue in the cluster is preferentially phosphorylated reflects a high degree of flexibility in that region. An important conclusion from this study was that autophosphorylation of CapB is expected to induce dissociation of the ring structure, by a conformational change that disrupts the amino acid contacts at the interaction interface, while maintaining the interaction between individual CapB-CapA couples. The two BY-kinase structures also provided a rationale for the substrate specificity of Tyr vs Ser/Thr kinases and the difference between BY-kinases and the structurally highly similar ATPases such as MinD [40].

The resolved structures of BY-kinases have rekindled interest in a decade-old question, why are there distinct *Proteobacteria*- and *Firmicutes*-type architectures; one with a single-polypeptide chain, and the other with two functional domains (transmembrane and kinase) split in separate proteins (Fig. 1). BY-kinases in *Proteobacteria* have a large extracellular (periplasmic) loop that allows them to interact with the polysaccharide translocation machinery [6], and this loop is significantly shorter in *Firmicutes* which lack the outer membrane and all corresponding structures. However, both types of BY-kinases are perfectly capable of phosphorylating various intra-cellular protein substrates, so this particular activity (substrate phosphorylation and recognition of multiple substrates) does not seem to be linked with any one of the two particular architectures. The structural study on CapB indicated that upon octamer dissociation, each individual BY-kinase molecule remains in contact with its transmembrane modulator CapA, and were it ever to separate from it, it would become effectively inactive as a kinase, due to destabilization of the ATP-binding site [36]. And yet the fact remains, BY-kinases in *Firmicutes* have evolved with an option to dissociate from their transmembrane modulators, which makes it rather difficult to imagine that they never do so. Our group has recently performed localization studies on the BY-kinase PtkA in *B. subtilis* using fluorescent protein fusions, and we were able to demonstrate that PtkA co-localizes with its transmembrane modulator TkmA under certain growth conditions, but we were equally able to identify growth conditions where PtkA dissociates from TkmA and becomes prevalently cytosolic (Jers & Mijakovic, unpublished results). In some cases PtkA also co-localized with some of its newly identified proteins substrates. We further demonstrated that PtkA can interact with other cytosolic proteins that are not its substrates but can modulate its kinase activity (Shi, Noirot-Gros & Mijakovic, unpublished results). This incited us to speculate that BY-kinases in *Firmicutes* might have a separated kinase domain in order to make it accessible to other modulators, and therefore participate in signaling pathways other than the principal one that starts at

the cell membrane, with the classical BY-kinase transmembrane domain. Arguments to support our hypothesis are admittedly not abundant at the moment, but we are confident that further studies in this direction will bring about another important turning point in the story of BY-kinases.

DETECTING PHOSPHO-TYROSINES: DEVELOPMENTS IN MASS SPECTROMETRY PHOSPHOPROTEOMICS

In *Eukarya*, protein-tyrosine phosphorylation only accounts for approximately 0.05% of the total cellular protein phosphorylation, even though almost one fifth of the cellular kinome encodes protein-tyrosine kinases [41]. Despite the average scarcity in terms of occupancy of phosphorylation sites, protein-tyrosine phosphorylation plays key roles in eukaryal signal transduction, cell differentiation and growth. In *Bacteria*, occupancy of protein-tyrosine phosphorylation sites is also very low, to the extent that this type of phosphorylation has not been identified at all by traditional proteomics approaches using 2D-gels and low-resolution mass spectrometry [42]. Only recently, the advent of gel-free analysis coupled to high-resolution mass spectrometry enabled us to systematically detect phospho-tyrosines in bacterial phosphoproteomes [27, 43-45]. In terms of the number of identified phosphorylation sites, phospho-tyrosines account for 3-10% of published bacterial phosphoproteomes and this is a considerable over-representation compared to the eukaryal systems. Nevertheless, in terms of actual site occupancy tyrosine phosphorylation remains very low, and the signals of tyrosine-phosphorylated peptides during mass spectrometry analysis are as a rule difficult to detect due to ion suppression effects from a comparatively high background of non-phosphorylated peptides. Therefore, enrichment techniques for phosphotyrosine-containing peptides preceding mass spectrometry analysis represent a promising area for improvement. In all phosphoproteomic studies performed in bacteria so far, phosphopeptides were enriched by methods not distinguishing between various phosphoesters (phospho-serine, -threonine and -tyrosine), such as using strong cationic exchange, titanium-oxide or immobilized metal affinity chromatography. Anti-phosphoamino acid antibodies against phospho-serine/threonine and to lesser extent against phospho-tyrosine are infamous for somewhat relaxed specificity, due to the fact that antibodies recognize a stretch of 10-11 amino acids and a single phosphoamino acid is not an ideal epitope. While this may represent a challenge for specific detection by Western blots, in terms of phosphopeptide enrichment from crude protein extracts antibodies can be a useful tool. In studies performed on eukaryal systems, the 4G10 and the PY100 antibodies were found to be the most efficient for the immune-affinity enrichment of phosphotyrosine proteins and peptides, and a combined use of the two antibodies was recommended to obtain the best result [46]. It seems therefore that a viable route towards global tyrosine phosphorylation studies in bacteria might imply antibody based enrichment prior to mass spectrometry analysis. In terms of cellular signaling, protein-tyrosine phosphorylation is a dynamic event and quantitative and time resolved analytical approaches are prerequisite to understanding its physiological role; even more so bearing in mind the short generation time of bacteria. In quantitative

phosphoproteomics, stable isotope labeling of cell cultures with amino acids (SILAC) provides reliable means of relative quantification of phosphotyrosines [47]. However, SILAC-based quantification imposes some limitations in terms of choice of growth medium. Alternative approaches rely on labeling after the growth phase, thus not influencing the growth conditions, such as for example the dimethylation of peptides with stable isotopes [48]. In parallel to mass spectrometry-based phosphoproteomics, unrelated approaches are being developed to study protein-tyrosine phosphorylation. They may lack the broadness of the global phosphoproteomic approaches, but may have other advantages in terms of time resolved detection of specific phosphorylation events. For example, electrochemical biosensors have been developed to monitor specific tyrosine kinase activities (and screening of inhibitors) *in vitro* [49]. In this particular study, the authors have used specific biotinylated substrate peptides immobilized on a streptavidin-coated indium-tin oxide electrode surface. The phosphorylation is detected using an ATP analogue adenosine gamma-thio triphosphate. After kinase-catalysed thio-phosphorylation of the immobilized peptides, the electrode surface was exposed to gold nanoparticles and the signals were thereafter detected by voltametry, using gold-chloride electrochemistry. This method seems particularly promising for *in vitro* screening of specific tyrosine-kinase inhibitors, and other developments in nanotechnology biosensors may hold a promise for applications allowing *in vivo* studies of signal transduction in microorganisms.

PERSPECTIVES

Site-specific bacterial phosphoproteomes are being published at an increasing pace [27, 43-45], and with the high accuracy mass spectrometry becoming more accessible we may reasonably expect to identify large numbers of BY-kinase substrates in various bacterial organisms. At present, bioinformatic predictors of protein phosphorylation sites are available. Neural-network algorithms, such as NetPhosBac [50], trained with available datasets on experimentally identified phosphorylation sites, can predict serine/threonine phosphorylation based on sequence homology and conservation of sites. Alternative predictors are the ones based on local structural disorder, such as DISPHOS [51]. At present, the number of identified sites of protein-tyrosine phosphorylation in bacterial proteins is not sufficient to envisage the construction of homology-based predictors such as NetPhosBac, but approaches based on local structural disorder seem more promising. In particular, weak conservation of bacterial phosphoproteomes on the phosphorylation site level is an argument in favour of developing clade-specific predictors, rather than global bacterial predictors. Upon examination of published tyrosine phosphorylation sites assigned to a specific kinase, as for example Ugd, TuaD, SsbA and SsbB proteins phosphorylated by PtkA in *B. subtilis* [21,28], no clear kinase recognition motif has emerged in the immediate surroundings of the phosphorylated residues. It is therefore plausible to assume that BY-kinases, much like their cognate phosphotyrosine-protein phosphatases [52], recognize structural motifs distant from the phosphorylated(able) residue on which they act. One big remaining question concerning the regulatory role of BY-kinases is the regulation of their kinase activity. BY-kinases described in recent structural

studies offer a snapshot of a constitutively active kinase, but we cannot be certain that this is indeed the case *in vivo*. The presence of large extracellular loops, by analogy to eukaryal systems, suggests the existence of extracellular signals that might trigger kinase activity. However, in *Proteobacteria* these extracellular loops are heavily involved in interactions with polysaccharide translocation machinery, and it is questionable whether they may form a real signal acceptor site. In bacteria in general, our knowledge on extracellular signals that trigger the activity of cytosolic serine/threonine and tyrosine kinases is very limited [53], but this may simply reflect our lack of investigative approaches concerning this part of the signaling cascade. The amount of data gathered on BY-kinases is at present not extensive enough to allow in-depth modeling of tyrosine-phosphorylation-based regulatory networks. However, the available elements on specific substrate activation should soon allow the construction of simple Boolean-type models, integrating all known BY-kinase interactants. Since tyrosine phosphorylation is a dynamic event; it may be justified to invest some efforts in quantitative, time-resolved analysis of BY-kinase cellular functions, and create dynamic models describing the behavior of these regulatory loops. These efforts may be particularly well justified by the relatively high connectivity of BY-kinases in Firmicutes, with a large number of substrates and alternative modulators.

Recent reviews concerning BY-kinases and bacterial protein phosphorylation in general [54,55] point out the promise they hold in terms of strategies for battling infective diseases caused by bacterial pathogens. The main advantage of BY-kinases in this respect is the fact that they have no eukaryal homologues, so their specific inhibitors are less likely to affect the host than for example the inhibitors of Hanks type serine/threonine kinases, which are present both in *Bacteria* and *Eukarya*. However, as discussed above, BY-kinases seem to be quite promiscuous in terms of choosing their substrates. Along these lines, Lin *et al.* [27] justifiably argue that site-specific knowledge concerning the substrates regulated by BY-kinases might provide more specific targets for inhibitory drugs. There is nevertheless an agreement in the field that inhibiting bacterial tyrosine phosphorylation is a promising venue in developing new therapeutics, and considerable efforts are already committed to this approach.

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