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Substrate Specificity of the *Bacillus subtilis* BY-Kinase PtkA Is Controlled by Alternative Activators: TkmA and SalA

Abderahmane Derouiche¹, Lei Shi¹, Aida Kalantari¹ and Ivan Mijakovic¹,²*

¹ Division of Systems and Synthetic Biology, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden, ² Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Lyngby, Denmark

Bacterial protein-tyrosine kinases (BY-kinases) are known to regulate different aspects of bacterial physiology, by phosphorylating cellular protein substrates. Physiological cues that trigger BY-kinases activity are largely unexplored. In *Proteobacteria*, BY-kinases contain a cytosol-exposed catalytic domain and a transmembrane activator domain in a single polypeptide chain. In *Firmicutes*, the BY-kinase catalytic domain and the transmembrane activator domain exist as separate polypeptides. We have previously speculated that this architecture might enable the *Firmicutes* BY-kinases to interact with alternative activators, and thus account for the observed ability of these kinases to phosphorylate several distinct classes of protein substrates. Here, we present experimental evidence that supports this hypothesis. We focus on the model *Firmicute*-type BY-kinase PtkA from *Bacillus subtilis*, known to phosphorylate several different protein substrates. We demonstrate that the transcriptional regulator SalA, hitherto known as a substrate of PtkA, can also act as a PtkA activator. In doing so, SalA competes with the canonical PtkA activator, TkmA. Our results suggest that the respective interactions of SalA and TkmA with PtkA favor phosphorylation of different protein substrates *in vivo* and *in vitro*. This observation may contribute to explaining how specificity is established in the seemingly promiscuous interactions of BY-kinases with their cellular substrates.

**Keywords:** bacterial protein-tyrosine kinase, protein phosphorylation, kinase specificity, kinase activator, transcription factor

**INTRODUCTION**

BY-kinases are bacterial enzymes found in roughly one half of sequenced bacterial species, but with no direct counterparts in *Eukarya* (Jadeau et al., 2008; Shi et al., 2014a). Their function is to regulate different cellular processes via phosphorylation of protein substrates. It is important to note that a single BY-kinase can phosphorylate several distinct proteins, and affect their respective roles. For example, the model BY-kinase PtkA from *Bacillus subtilis* regulates the activity of several enzymes (Mijakovic et al., 2003; Petranovic et al., 2009; Jers et al., 2010) and protein chaperones (Shi et al., 2016). In addition, PtkA has a role in regulating biofilm development (Kiley and Stanley-Wall, 2010; Gerwig et al., 2014), DNA
metabolism (Petranovic et al., 2007), and sub-cellular localization of proteins (Jers et al., 2010). Finally, PtkA also phosphorylates transcriptional regulators and controls their binding to DNA target sequences (Derouiche et al., 2013, 2015). For a global overview of various regulatory roles of BY-kinases, several reviews are available (Grangeasse et al., 2007, 2012; Chao et al., 2014; Mijakovic and Deutscher, 2015; Mijakovic et al., 2016). In Proteobacteria, such as Escherichia coli, BY-kinases are large transmembrane proteins. They contain a catalytic domain exposed to the cytosol, and an activator domain, typically with a flexible extracellular loop flanked by two transmembrane helices (Douplet et al., 2002). In Firmicutes, such as B. subtilis, the BY-kinase domain is in most cases a self-standing protein that engages in protein–protein interaction with a separate transmembrane activator (Mijakovic et al., 2003). The Firmicute-type BY-kinase and activator pairs are typically encoded by neighboring genes, and this has sparked a long-standing debate on whether the prototype BY-kinase was of the Firmicute (“split”) or Proteobacteria (“joint”) type (Grangeasse et al., 2007; Shi et al., 2010). We have previously speculated that the rationale for the “split” type architecture could be to enable the BY-kinase domain to interact with more than one activator (Shi et al., 2010). First evidence in this direction came from the study by Shi et al. (2014c), demonstrating that autophosphorylation of PtkA can be stimulated in the presence of the cell division protein MinD (2014c), demonstrating that autophosphorylation of PtkA can be stimulated in the presence of MinD.

That study had, however, not established whether MinD–PtkA interaction is required for PtkA autophosphorylation. Here we provide evidence in this direction came from the study by Shi et al. (2014c). That study had, however, not established whether MinD–PtkA interaction is required for PtkA autophosphorylation. Our results point to the existence of different classes of PtkA substrates: those phosphorylated preferentially in the presence of TkmA, those phosphorylated preferentially in the presence of SalA, and those phosphorylated equally in the presence of either activator. This finding could be a useful basis for deciphering the specificity in complex interaction networks of BY-kinases and their substrates.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

For gene cloning, E. coli NM522 was used. The strain E. coli M15, carrying pREP4-GroESL (Amrein et al., 1995), was used for biosynthesis of tagged proteins. For in vivo mutant construction, B. subtilis BS514 (B. subtilis 168 trp+ Pr: neoR) was used. All mutants used in this work are listed in the Supplementary Table S1. B. subtilis and E. coli strains were grown in Luria-Bertani (LB) medium with shaking, at 37°C. When relevant, ampicillin (100 μg/ml) and kanamycin (25 μg/ml) for E. coli and erythromycin (1 μg/ml), neomycin (5 μg/ml), and phleomycin (2 μg/ml) for B. subtilis were added to the medium.

**DNA Manipulation and Strain Construction**

All PCR primers with restriction enzymes are listed in Supplementary Table S2. The deletion of tkmA gene was performed using the modified mutation delivery method of Fabret et al. (2002). The deletion of tkmA gene was made using the pairs of primers ΔtkmA-ext fwd/ΔtkmA-ext rev were used. To introduce an in-frame deletion between the codons 10 and 240 of the gene tkmA, partially complementary primers ΔtkmA fwd/ΔtkmA rev were used. Gene fatR was amplified by PCR from B. subtilis 168 genomic DNA and inserted into the vector pSG1729 (Lewis and Marston, 1999) between the restriction enzymes site KpnI and XhoI, which results in the replacement of the gfp gene by Strep-fatR. B. subtilis wild type (WT). ΔsalA (Derouiche et al., 2015), ΔpikA (Jers et al., 2010), and ΔtkmA strains were transformed with the pSG1729-fatR construct and selected for erythromycin resistance. All constructs were sequenced to check for absence of unsolicited mutations.

**Synthesis and Purification of Tagged Proteins**

SalA, Ugd, Asd, and FatR proteins were produced in E. coli M15 as 6x His N-terminal fusions. The cultures were grown with shaking (200 rpm) at 37°C until OD600 of 0.5. To induce the expression, 1 mM of isopropyl β-D-1 thigalactopyranoside (IPTG) was added to the medium, and the cultures were kept in the same growth conditions for three more hours. To purify the 6x His-tagged proteins, Ni-NTA columns were used (Qiagen) as described previously (Mijakovic et al., 2003). The Step- tagged FatR protein was purified from B. subtilis BS514 using the Strep Tactin affinity chromatography (Novagen) as described in our previous study (Shi et al., 2014b). The purity of proteins was checked by SDS-PAGE and aliquots of all purified proteins were kept at −80°C in buffer composed of 50 mM Tris-Cl pH 7.5, 100 mM NaCl and 10% glycerol.
In vitro Protein Phosphorylation Assays

All in vitro phosphorylation assays were performed in the presence of 50 µM ATP \([\gamma-\text{32P}]\), which corresponds to 20 µCi/mmol of labeled ATP. A standard 40 µl reaction mix contained 1 µM BY-kinase PtkA and 1 µM activator proteins (TkmA-NCter or SalA). Concentration of protein substrates was 5 µM, and the reaction medium contained 1 mM MgCl₂ and 100 mM Tris-HCl (pH 7.5). After 1 h of incubation at 37°C, the reactions were stopped by adding SDS-PAGE loading dye and heating for 5 min at 100°C. The proteins were separated by electrophoresis using SDS-PAGE. The gels were washed and then boiled in 0.5 M HCl for 10 min, and transient staining with Coomassie Blue was used to identify protein bands. The gels were dried overnight and the autoradiography signals of phosphorylated proteins were revealed by a PhosphoImager (FUJI). The experiments were performed in triplicates, each time with protein aliquots purified independently. One representative assay is shown for each reaction.

Asd and Ugd Assay in Crude Extracts

For enzymatic assays, B. subtilis strains were grown in LB with vigorous shaking in flasks at 37°C. Samples were harvested at the mid-exponential phase (OD₆₀₀ = 0.4). Cells were lysed by sonication. The lysis buffers (composition adapted for each assay) contained 1% tyrosine phosphatase inhibitor cocktail (Sigma) and 1 mg/ml lysozyme (Sigma). For the Ugd assay, the buffer contained 50 mM 3-(N-morpholino) propanesulfonic acid (pH 7.0), 200 mM KCl, 0.1 mM EDTA, and 10 mM 2-mercaptoethanol. After centrifugation, the supernatant was desalted on PD-10 columns (GE healthcare), to remove all metabolites and cofactors. Total centrifugation, the supernatant was desalted on PD-10 columns (GE healthcare). In 0.5 Tris-acetate-EDTA using non-denaturing gels (12% polyacrylamide) and heating for 5 min at 100°C. The proteins were separated by electrophoresis using SDS-PAGE. The gels were washed and then boiled in 0.5 M HCl for 10 min, and transient staining with Coomassie Blue was used to identify protein bands. The gels were dried overnight and the autoradiography signals of phosphorylated proteins were revealed by a PhosphoImager (FUJI). The experiments were performed in triplicates, each time with protein aliquots purified independently. One representative assay is shown for each reaction.

Electrophoretic Mobility Shift Assays

Strep-tagged FatR was purified from B. subtilis in different backgrounds: WT, ΔsalA, and ΔtkmA. The DNA binding assay was performed with a 24 bp double-stranded DNA containing the fatR operator sequence, as described by Derouiche et al. (2013). The molar ratio of the DNA probe to strep-tagged FatR proteins is indicated in the figure legend. The reaction mixtures were incubated for 1 h at the room temperature. The migration was performed for 2 h at 2 V/cm in 0.5 Tris-acetate-EDTA using non-denaturing gels (12% polyacrylamide). The experiment was repeated three times with independently purified proteins. One representative experiment is shown.

RESULTS AND DISCUSSION

SalA Is an Activator of PtkA

Recently, we have established that the C-terminus of SalA can interact directly with the BY-kinase PtkA, which results in phosphorylation of the SalA residue tyrosine 327. This phosphorylation enhances its function as a repressor of scoC (Derouiche et al., 2015). Interestingly, the in vitro phosphorylation of SalA took place in absence of the canonical activator TkmA (Derouiche et al., 2015). This finding is difficult to reconcile with the available structural data. The BY-kinases from Firmicutes require the interaction with TkmA-type activators to stabilize the ATP binding pocket in their active site in order to autoprophosphorylate or phosphorylate substrates (Olivares-Illana et al., 2008). The presence of TkmA is a strict requirement for phosphorylation of all previously characterized PtkA substrates (Mijakovic et al., 2003, 2006; Jers et al., 2010; Derouiche et al., 2013, 2015; Shi et al., 2016). The first clue in explaining this observation comes from the structural homology of SalA with BY-kinases and MinD proteins (Derouiche et al., 2016). A part of the C-terminal region of SalA which interacts with PtkA shows sequence homology (by circular permutation) with the activating fragment of Staphylococcus aureus BY-kinase activator CapA and the N-terminus of its cognate BY-kinase CapB (Olivares-Illana et al., 2008). This suggested that the C-terminus of SalA could be expected to activate PtkA. To investigate this, we performed a series of PtkA autophosphorylation reactions, keeping the kinase concentration constant and varying the concentration of SalA (Figure 1A, lanes 2–5). The in vitro phosphorylation assay demonstrated that the presence of SalA strongly stimulates autophosphorylation of PtkA in the absence of its canonical activator TkmA.
SalA and TkmA act as Alternative Activators of PtkA and Can Direct the Activity of the Kinase Toward Different Substrates

The results presented in the previous section establish SalA as a bona fide candidate for an alternative activator of PtkA. If PtkA can interact with either of the two activators in vivo, and the activation effect is similar, what is the reason for the existence of two activators? To address this question, an in vitro phosphorylation assay with purified proteins was performed, where we compared the ability of PtkA to phosphorylate several characterized protein substrates in the respective presence of either TkmA or SalA (Figure 2). The first characterized PtkA substrate, Ugd (Mijakovíc et al., 2003; Petranovic et al., 2009), was phosphorylated in the presence of either of the activators. By contrast, SalA promotes the phosphorylation of the PtkA substrate Asd more efficiently (Jers et al., 2010), while TkmA promoted the phosphorylation of the PtkA substrate FatR more...
SalA efficiently (Derouiche et al., 2013). When SalA served as an activator in the *in vitro* reaction, for example in the reaction with Asd and Ugd, phosphorylation of SalA was abolished (Figure 2B, lanes “Asd” and “Ugd”). This could mean that the PtkA-SalA interaction exists in two different and mutually exclusive modes: kinase-substrate and kinase-activator. It would be very interesting from the structural and functional perspective to explore this possibility in the future.

The hypothesis that SalA and TkmA may determine the choice of substrate preferentially phosphorylated by PtkA was next assessed *in vivo*. We used the same three PtkA substrates: Asd (preferentially phosphorylated in the presence of SalA), FatR (preferentially phosphorylated in the presence of TkmA), and Ugd (equally phosphorylated in the presence of either TkmA or SalA). PtkA-dependent phosphorylation is known to lead to a measurable modification of activity of all these substrates: increase of the Asd enzymatic activity (Jers et al., 2010), decrease of the Ugd enzymatic activity (Mijakovic et al., 2003; Petranovic et al., 2009). To test the impact of SalA and TkmA on the phosphorylation of PtkA substrates *in vivo*, we constructed strains with seamless deletions of *salA* and *tkmA*.

The Asd activity was measured directly in desalted crude extracts with phosphatase inhibitors (to preserve the Asd phosphorylation state). Compared to the WT, the activity was significantly reduced in the ΔptkA and ΔsalA strains, but not in ΔtkmA (Figure 3A). This indicated that Asd *in vivo* activation via phosphorylation depends on PtkA and SalA, but not on TkmA. For assessing FatR activity, we prepared *B. subtilis* strains expressing Strep-tagged FatR in the same relevant genetic backgrounds: WT, ΔsalA, and ΔtkmA. FatR was purified in the presence of phosphatase inhibitors and assayed for its ability to bind its target DNA (Figure 3B). The binding was not detectable with FatR purified from the WT and ΔsalA strains, but was detectable with the equal amount of FatR purified from the ΔtkmA strain, indicating that the absence of TkmA coincided with less efficient phosphorylation *in vivo*. Finally, for Ugd we also used the enzyme activity assay with desalted crude extracts (with phosphatase inhibitors) (Figure 3C). In this case the activity was reduced in ΔptkA compared to the WT,
but was not significantly affected in either ΔsalA or ΔtkmA, suggesting that in this case the two activators can complement each other’s absence. Thus all the in vivo and in vitro findings were in agreement, suggesting that SalA and TkmA can indeed play the role of alternative PtkA activators, with a potential to preferentially direct the kinase activity to certain substrates. Interestingly, our two-hybrid interaction assay had previously revealed that TkmA interacts with PtkB, a second BY-kinase from B. subtilis (Shi et al., 2014c). It is therefore tempting to speculate that PtkB might also have two alternative activators: TkmA and TkmB.

A Mutual Regulatory Loop between PtkA and SalA

Our findings indicate that there exists a mutual regulation between B. subtilis PtkA and SalA, which critically depends on the physical interaction between the C-terminal region of SalA and the catalytic domain of PtkA (Figure 4). This interaction has regulatory consequences for both proteins. In contact with PtkA substrates, SalA activates the kinase but does not get phosphorylated itself. In the absence of other substrates, as described previously (Derouiche et al., 2015), SalA gets phosphorylated by PtkA, this promotes its ATP binding, and in turn stimulates binding to its target DNA sequence, upstream of scoC. Accounts of such mutual regulatory loops are not very common in the literature. One example of negative feedback involves the human ubiquitin E3 ligase SIAH2 which is phosphorylated and activated by the serine/threonine kinase DYRK2. In turn, SIAH2 destabilizes and promotes degradation of the kinase (Pérez et al., 2012). Forward-feeding activation loops are exemplified by the murine Src family kinases, which activate the neurotrophin receptor tyrosine kinase TrkB, which in turn activates the kinases (Huang and McNamara, 2010). To the best of our knowledge, this is the first report of a mutual regulation loop based on a direct protein-protein interaction in bacteria. The case is particularly interesting since the interactants share a common evolutionary origin (Derouiche et al., 2016), suggesting that the interaction of SalA and PtkA with mutual regulatory consequences was maintained in the process of divergent evolution. One may expect that such cases of mutual regulation will turn out not to be uncommon in bacteria, since the reports on “moonlighting” proteins which have more than one known function are steadily accumulating (Jeffery, 1999; Wang et al., 2013; Wang and Jeffery, 2016).

CONCLUSION

The findings presented here suggest that the access of PtkA to alternative activators: TkmA, SalA or MinD, could determine when and where PtkA acts, and which substrates it phosphorylates. We have previously shown that overexpression of TkmA confines PtkA to the membrane (Jers et al., 2010) and that MinD is capable of recruiting PtkA to the cell pole (Shi et al., 2014c). Therefore, the sub-cellular localization of PtkA may indeed be dynamic, and respond to expression levels or the availability of alternative activators (Figure 4). So far no specific substrates associated with the PtkA-MinD complex have been demonstrated. However, their existence is probable, since MinD was shown to activate PtkA autophosphorylation (Shi et al., 2014c). The hypothesis of PtkA cycling among different activators may be an answer to the long-standing question about the rationale behind the Firmicute-type “split” architecture of BY-kinases. Our speculation is that Firmicutes employ a separate BY-catalytic domain in order for it to be able to interact with more alternative activator proteins. These interactions in turn lead to the capacity to differentially interact with, and phosphorylate, a larger number of different cellular substrates (Figure 4). The final consequence is an increased level of complexity in the BY-kinase-substrate network. In order to provide a definite proof for this hypothesis, our analysis has to be extended to all known substrates of PtkA. An attempt will have to be made to quantify the in vivo levels of phosphorylation of all substrates in the ΔptkA, ΔsalA, and ΔtkmA knockouts using mass spectrometry proteomics. The hypothesis that SalA interacts with PtkA in two different modes: kinase-substrate and kinase-activator, will also have to be examined by structural analyses. While, we commit to the pursuit of these analyses outside the scope of the present report, we deem it relevant to notify the scientific community that alternate activators of BY-kinases exist, and can affect substrate specificity of these particular enzymes.

AUTHOR CONTRIBUTIONS

AD, AK, and LS performed the experiments. AD and IM analyzed the data. AD and IM wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01525

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