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Review Article

REVIEW ARTICLE

Protein phosphorylation and its role in archaeal signal transduction

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One sentence summary: The authors review the current knowledge about protein phosphorylation in Archaea and its impact on signaling in this organism group.

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ABSTRACT

Reversible protein phosphorylation is the main mechanism of signal transduction that enables cells to rapidly respond to environmental changes by controlling the functional properties of proteins in response to external stimuli. However, whereas signal transduction is well studied in Eukaryotes and Bacteria, the knowledge in Archaea is still rather scarce. Archaea are special with regard to protein phosphorylation, due to the fact that the two best studied phyla, the Euryarchaeota and Crenarchaeaota, seem to exhibit fundamental differences in regulatory systems. Euryarchaeota (e.g. halophiles, methanogens, thermophiles), like Bacteria and Eukaryotes, rely on bacterial-type two-component signal transduction systems (phosphorylation on His and Asp), as well as on the protein phosphorylation on Ser, Thr and Tyr by Hanks-type protein kinases. Instead, Crenarchaeota (e.g. acidophiles and (hyper)thermophiles) only depend on Hanks-type protein phosphorylation. In this review, the current knowledge of reversible protein phosphorylation in Archaea is presented. It combines results from identified phosphoproteins, biochemical characterization of protein kinases and protein phosphatases as well as target enzymes and first insights into archaeal signal transduction by biochemical, genetic and polyomic studies.

Keywords: Archaea; Crenarchaeota; Euryarchaeota; reversible protein phosphorylation; protein kinase; protein phosphatase; regulation; signal transduction

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INTRODUCTION

Archaea

The third domain of life, Archaea, has been established in the 1970s–1990s by Carl Woese and colleagues (Woese, Kandler and Wheelis 1990) based on their phylogenetic studies on small subunit ribosomal RNA (rRNA). Initially Archaea were categorized as extremophiles, which thrive in hostile environments characterized by extremes of temperature, pH, salt or combinations thereof, or organisms with unique metabolic traits, i.e. methanogens. Today, using environmental molecular biology approaches, it is well accepted that Archaea are ubiquitous and also widely distributed in moderate habitats and play a major role in geochemical cycles (Delong 1998; DeLong and Pace 2001). But still, to date, most of the cultivated species are extremophiles.

Today 1237 fully sequenced archaeal genomes are available (as of May 2016) (Genome Online Database [GOLD] [http://genomesonline.org/]) and six major archaeal phyla have been proposed: Euryarchaeota, Crenarchaeota, Nanoarchaeota, Thaumarchaeota, Aigarchaeota (candidate phylum) and Korarchaeota. The largest and first established phyla were the Euryarchaeota and Crenarchaeota and more recently four of them (Thaumarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota) were combined to the 'TACK' superphylum (Guy and Ettema 2011).

At a first glance, Archaea resemble Bacteria, the second prokaryotic lineage, in respect to their unicellular lifestyle, the lack of organelles and nucleus, cell shape and size. Also they possess a similar DNA structure with one circular DNA, plasmids and operon structures are commonly found. In regard to metabolic complexity, Archaea resemble Bacteria and lower Eukaryotes. Absolutely unique for the Archaea is the absence of murein, one of the key features of bacterial cell walls, and their membrane. The archaeal membrane lipids are composed of isoprenoid chains ether-linked to *sn*-glycerol 1-phosphate head groups (i.e. dibiphytanyltetraethers or biphytanylethers) rather than fatty acids ester-linked to *sn*-glycerol 3-phosphate, as found in *Bacteria* and *Eukaryotes* (Albers and Meyer 2011).

Intriguingly, related to information processing, the processes involved in transformation of DNA to protein (e.g. replication, transcription, translation, repair) in Archaea resemble their respective eukaryotic counterparts, but are often less complex (Bell and Jackson 1998; Bell, Magill and Jackson 2001; Soppa 2001; Hickey, Conway de Macario and Macario 2002; Geiduschek and Ouhammouch 2005; Grohmann and Werner 2011). In line with this similarity, the recent identification of Lokiarchaeota, as a missing link between Archaea and Eukaryotes, promotes the direct descent of Eukaryotes from the archaeal TACK superphylum. Thus, a new two domain tree of life with Bacteria on one side and on the other side Archaea with the Eukaryotes emerging as a monophyletic group within the Archaea is currently discussed and is a matter of scientific debate (Guy and Ettema 2011; Raymann, Brochier-Armanet and Gribaldo 2015). In the context of the shared ancestry of Archaea and Eukaryotes and the similarities found in DNA metabolism, Archaea are especially interesting targets to study regulation by post-translational modification (PTM) and mechanisms of signal transduction.

PTMs in Archaea

PTMs enable organisms to respond rapidly to changing environmental conditions, such as depletion of nutrients or changes of abiotic factors such as temperature. This allows cells to change the properties of their current proteome in a way that ensures adaptation and suits their current lifestyle best, without relying on the synthesis of new proteins.

In 2011, Khoury, Baliban and Floudas (2011) performed a study where they curated the SWISS-Prot database regarding PTMs, which were either identified experimentally (via proteomics) or predicted in all three domains of life. They were able to identify more than 300 000 PTMs subcategorized to 28 different types of PTMs across 5605 different organisms. Of these PTMs, phosphorylation was the most abundant modification (~140 000 modifications). Only 305 (0.13%) phosphorylation sites were identified in the 49 archaeal species available at that time. This study illustrates nicely that the investigation of PTMs in Archaea is still in its infancy.

Nevertheless, several different PTMs have been reported in Archaea. Among these are many PTMs that are also present in Bacteria and Eukaryotes like phosphorylation, acetylation, N-and O-glycosylation and methylation (summarized in Table 1). In Archaea, the ubiquitin-like modification SAMPylation (small archaeal modifier protein), which targets proteins for the proteasomal degradation, was identified in *Haloferax volcanii* (Humbard *et al.* 2010). Recently, the small protein modification, urmylation, which was only reported in Eukaryotes so far, was found in *Sulfolobus acidocaldarius* (Anjum *et al.* 2015). Furthermore, Archaea also possess amino acid modifications like hypusination and thiolation (Eichler and Adams 2005).

Generally, PTMs found in Bacteria and Eukaryotes can also be found in Archaea. Many archaeal PTMs are rather similar to their eukaryotic counterpart like the use of a dolichyl pyrophosphate carrier in N-glycosylation and urmylation. However, here we want to focus on phosphorylation and its role in archaeal signal transduction.

Reversible protein phosphorylation

One of the best-studied PTMs in all three domains of life is reversible protein phosphorylation, which plays a major role in signal transduction and is involved in the regulation of nearly all processes within the cell. Protein phosphorylation and dephosphorylation is a covalent, reversible modification of amino acids that involves protein kinases (PKs) and protein phosphatases (PPs). PKs catalyze the phosphorylation, i.e. the transfer of the γ-phosphate group from nucleoside triphosphates (usually adenosine triphosphate (ATP)) to other proteins, whereas PPs remove the covalently linked phosphate group from the phosphorylated protein (phosphoprotein) by hydrolysis (dephosphorylation). Therefore, reversible protein phosphorylation is capable of regulating the properties of proteins rapidly and thus allows for quick responses to external stimuli (Kennelly 2003).

Protein phosphorylation was originally discovered in the 1950s by Krebs and Fischer during their investigation of the rabbit skeletal muscle (Krebs and Fischer 1956). They demonstrated that phosphorylase B is converted to phosphorylase A via autophosphorylation in the presence of [γ-^{32}P]ATP. It took 22 years until PK activity was demonstrated in the Gram-negative bacteria *Salmonella typhimurium* (Wang and Koshland 1978) and *Escherichia coli* (Manai and Cozzone 1979). One year later, the identification of the first prokaryotic target protein, isocitrate dehydrogenase, was reported in *E. coli* (Garnak and Reeves 1979). Only a short time later protein phosphorylation was reported in the Archaea, i.e. the Euryarchaeon *Halobacterium salinarium* (Spudich and Stoeckenius 1980), leading to the discovery of the first

Table 1. Overview of some important PTM in all three domains of life.

PTM	Archaea	Bacteria	Eukarya	References
Phosphorylation				
His/Asp, TCS	Yes (not Crenarchaeota)	Yes	Yes	Ashby (2006); Kobir *et al.* (2011); Pereira, Goss and Dworkin (2011); Shi *et al.* (2014); Hanks and Hunter (1995); Kennelly (2014)
Ser/Thr	Yes (Hanks type)	Yes (Hanks type)	Yes (Hanks type)	
Tyr	Yes (Hanks type)	Yes (Walker type)	Yes (Hanks type)	
Glycosylation				
N-linked	Yes (dolichyl mono- or pyrophosphate carrier)	Yes (only in some δ- and ε-proteobacteria, undecaprenyl pyrophosphate carrier)	Yes (dolichyl pyrophosphate carrier)	Dell *et al.* (2010); Nothaft and Szymanski (2010); Jarrell *et al.* (2014); Schwarz and Aebi (2011)
O-linked	Yes	Yes	Yes	
Acetylation				
N-terminal	Yes (rare in methanogens)	Yes	Yes	Soppa (2010); Ouidir, Kentache and Hardouin (2016)
Protein internal (Lys ε-amino group)	Yes	Yes	Yes	
Methylation	Yes	Yes	Yes	Bedford and Richard (2005); Lanouette *et al.* (2014)
Small protein modifications				
Ubiquitination	No	No	Yes	Maupin-Furlow (2011, 2013a,b, 2014); Striebel *et al.* (2014); Anjum *et al.* (2015); Jüdes *et al.* (2015)
Pupylation	No	Yes	No	
SAMPylation	Yes	No	No	
Urmylation	Yes	No	Yes	

Selected references about the respective PTM are listed in the table, more general reviews related to PTMs in the three domains of life are as follows: Cain, Solis and Cordwell (2014); Eichler and Adams (2005); Maupin-Furlow (2013b); Walsh, Garneau-Tsodikova and Gatto (2005); Mann and Jensen (2003).

archaeal two-component system (TCS), CheA and CheY (Rudolph *et al.* 1995; Falke *et al.* 1997). In the 1980s, Skorko reported the presence of protein phosphorylation in the thermoacidophilic Crenarchaeon *S. acidocaldarius* (Skorko 1984, 1989). He determined the PK activity in crude extracts of cells harvested in both the exponential and stationary growth phases and demonstrated higher PK activity in the stationary growth phase.

Until now, protein phosphorylation has been identified on His, Asp, Ser, Thr, Tyr, Cys, Lys and Arg residues (Matthews 1995; Khoury, Baliban and Floudas 2011; Mijakovic, Grangeasse and Turgay 2016) and can be categorized into different regulatory systems. The first one comprises phosphorylation of the positive charged amino acid His and the negatively charged Asp (for detailed discussion, see below). The phosphorylation of histidine forms a phosphoramidate (P–N) bond and of aspartate a mixed anhydride or acyl phosphate bond, both representing relatively high-energy bonds. The second system, phosphorylation of the polar amino acids Ser, Thr and Tyr results in a more stable phosphoester. Cys phosphorylation leads to a high-energy phosphorothiolate (P–S) bond and was observed for example as an intermediate in the bacterial phosphoenolpyruvate-dependent phosphotransferase system (Mijakovic and Macek 2012; Deutscher *et al.* 2014) or as part of the enzyme mechanism in protein-tyrosine phosphatases (Fuhrmann *et al.* 2009; Buchowiecka 2014). Phosphorylation of the positive charged amino acid Arg and Lys forms a high-energy phosphoramidate bond. Arg phosphorylation is used in eukaryotic cells for ATP formation from ADP to buffer the energy charge (ATP/ADP ratio) in some cells (Ellington 2001). In the Gram-positive bacterium, *Bacillus subtilis* arginine phosphorylation was shown to have an regulatory role and Arg PKs, p-Arg PP as well as the p-Arg proteome of *B. subtilis* was investigated in significant detail (Fuhrmann *et al.* 2009; Elsholz *et al.* 2012). Lysine phosphorylation is not well studied and there are only few reports available (Matthews 1995; Mijakovic, Grangeasse and Turgay 2016). In Archaea, until now only phosphorylation on His and Asp as well as Ser, Thr, and Tyr has been reported. This will be the focus of this manuscript.

Therefore, protein phosphorylation is well established in all three domains of life. In the past, the hypothesis was postulated that protein phosphorylation on Ser, Thr and Tyr residues is a typical eukaryotic feature, whereas Bacteria rely on His and Asp phosphorylation. However, today it is well known that His/Asp phosphorylation is also present in Eukaryotes and that protein phosphorylation on Ser, Thr and Tyr takes place in all three domains of life, i.e. Archaea, Bacteria and Eukaryotes (Koretke *et al.* 2000; Casino, Rubio and Marina 2010; Wuichet, Cantwell and Zhulin 2010; Pereira, Goss and Dworkin 2011; Schaller, Shiu

and Armitage 2011; Burnside and Rajagopal 2012; Capra and Laub 2012; Shi *et al.* 2014; Dworkin 2015). Interestingly, comparative genome analyses were unable to identify any His kinases and response regulators (RRs) within the Crenarchaeota and Nanoarchaeota, whereas several homologs were found in the Euryarchaeota (Eichler and Adams 2005; Ashby 2006; Galperin 2006, 2010).

In this review, we summarize the current knowledge of protein phosphorylation in Archaea, including the findings from phosphoproteome studies, the description of characterized bacterial-type TCS and Hanks-type PKs (also named eukaryotic-like PKs, ePKs) and PPs. We also present the results of genetic approaches that enabled the analysis of the first archaeal signal transduction cascades.

PHOSPHOPROTEINS AND PHOSPHOPROTEOME STUDIES IN ARCHAEA

Despite phosphoproteins being reported in Archaea already in the 1980s (Spudich and Stoeckenius 1980; Skorko 1984, 1989) and the identification of several phosphoproteins later on (for summary, see Table 2), it took until the 2000s for the first phosphoproteome studies to be carried out in the Archaea. The first genome-wide approach was performed in a *Halobacterium salinarium* (strain R1) wild-type (WT) and serine/threonine phosphatase (*serB*) deletion strain (Aivaliotis *et al.* 2009) using TiO_2 enrichment and liquid chromatography/mass spectroscopy. In total, 90 unique phosphopeptides from 69 *H. salinarum* proteins were identified and 81 phosphorylation sites were determined with a Ser/Thr/Tyr ratio of 86/12/1%. In accordance with the deletion of the *serB* gene, a 3-fold increase in serine phosphorylation in comparison to WT was observed.

Later on, a precursor acquisition independent of ion count (PACiFIC) approach was used to analyze the phosphoproteins in *Sulfolobus solfataricus* cells grown on either D-glucose or tryptone (Esser *et al.* 2012). In this study, a total of 1318 phosphorylation sites located on 690 phosphopeptides from 540 unique proteins were identified. Notably, a high preference on tyrosine phosphorylation was detected with a Ser/Thr/Tyr ratio of 26/21/54%. The identified phosphoproteins belong to almost all functional classes (21 out of 26 archaeal Clusters of Orthologous Genes (arCOGs)) supporting an essential role of protein phosphorylation in most cellular processes in *S. solfataricus*. The study focused on changes of phosphorylation patterns in the central carbohydrate metabolism in response to the offered carbon source. It revealed a significant role of protein phosphorylation in the control of central carbohydrate metabolism and channeling of the carbon flux in different metabolic pathways.

A second PACiFIC study in *S. acidocaldarius* enabled further insights into the importance of protein phosphorylation in Archaea (Reimann *et al.* 2013). In this study, the *in vitro* and *in vivo* functions of the only two phosphatases, Saci-PTP and Saci-PP2A, were analyzed by biochemical characterization as well as genetic and polyomics approaches. In *S. acidocaldarius* (parental strain, MW001) as well as the two PP deletion strains in total 801 unique phosphoproteins (1206 phospho-peptides) were identified again with an unusually high number of phosphorylated Tyr residues (pSer/pThr/pTyr % ratio of 35.6/28.1/36.2). Like in the previous study performed in *S. solfataricus*, phosphoproteins were identified in almost all arCOGs and 18 transcriptional regulators were found to be phosphorylated, among others ArnR1 the positive regulator of the archaellum operon (see below) as well as 5 of the predicted serine/threonine PKs of *S. acidocaldarius* (Reimann *et al.* 2013).

Therefore, as shown within Bacteria and Eukaryotes, a major number of proteins are phosphorylated within Archaea. This highlights the role for reversible protein phosphorylation in this domain of life. The broad distribution in almost all arCOG categories underlines the global regulatory function of protein phosphorylation. Unusually, a high number of tyrosine phosphorylation was identified in *Sulfolobus* sp. This feature has not been reported for hyperthermophilic Bacteria (i.e. *Thermus thermophilus;* Takahata *et al.* 2012) or the mesophilic Euryarchaeon *H. salinarium* strain R1 (Aivaliotis *et al.* 2009). Notably the two latter studies used TiO_2 enrichment strategies and in both organisms bacterial-type TCS are present in addition to phosphorylation on Ser, Thr and Tyr.

HIS AND ASP PHOSPHORYLATION (TCSs) IN EURYARCHAEOTA

Phosphorylation on His and Asp residues is found in a specific type of regulatory system, the TCS. These systems are present in all three domains of life (Koretke *et al.* 2000). The classical TCS consists of a His sensor kinase (HisK) and a response regulator (RR). The HisK is usually membrane bound and consists of two domains, a sensor input domain (extracellular) and a His kinase, transmitter domain (intracellular), whereas the RR is usually a cytoplasmic protein. The sensor input domain is stimulated via an environmental signal (e.g. a small molecule ligand), which leads to the activation of the HisK domain. Within the HisK domain, a specific His residue is then autophosphorylated and the phosphoryl group is subsequently transferred to an Asp residue of the RR receiver domain. The phosphorylation of the RR leads to the activation of its output domain, which triggers the corresponding cellular response, i.e. phosphorylation of the target protein (Fig. 1). TCSs were originally discovered in Bacteria by several independent studies in 1985 and 1 year later the name 'two-component regulatory system' was introduced by Nixon, Ronson and Ausubel (1986). Today, it is known that TCSs also exist in the genomes of Euryarchaeota and in certain non-animal Eukaryotes (e.g. fungi, plants) (Alex and Simon 1994; Loomis, Shaulsky and Wang 1997; Lohrmann and Harter 2002; Schaller, Shiu and Armitage 2011). As mentioned above, the exception within the Archaea are the Nanoarchaeota and Crenarchaeota (Ashby 2006; Galperin 2006, 2010). Both phyla comprise only (hyper)thermophilic microorganisms and possess no TCSs.

Apart from these classical two-step phosphorelay TCS, hybrid kinases (four-step phophorelay) represent a common modification in Prokaryotes and Eukaryotes. In hybrid kinases, a receiver domain of a RR is directly fused to the sensor kinase (HisK) and therefore autophosphorylation and phosphotransfer occur within the same protein. Subsequently, the phosphoryl group is further transferred via a histidine phosphotransferase to the receiver domain of a second RR (Fig. 1). However, to date, there are no phosphorelays studied in the Archaea. Therefore, we will not discuss these further in this review.

Furthermore, there is a third system, the one-component system (OCS). The detailed analysis of 145 prokaryotic genomes by Ulrich, Koonin and Zhulin (2005) revealed that OCSs (~17 000) are more abundant in the investigated genomes than TCSs (~4000). In OCSs, the sensor input domain is directly fused to the output domain in a single protein. They lack the histidine kinase domain of the HisK and the receiver domain of the RR and thus no protein modification is involved in OCSs. Typical examples are transcriptional regulators that contain a ligand-binding domain and DNA-binding helix-turn-helix (HTH) domain. Analysis of transmembrane regions in this study predicted that OCSs are

Table 2. Characterized phosphoproteins from archaeal species. Updated table adapted from Esser *et al.* (2012).

Accession Nr.	Target protein	Predicted function	Organism	Information	Reference
Q973R9	ST0829	FHA domain-containing protein	*Sulfolobus tokodaii*	Phosphorylated by ST1565	Wang *et al.* (2010)
–	–	Methyltransferase activating protein	*Methanosarcina barkerii*	Autophosphorylation with ^{32}P	Daas *et al.* (1996)
Q76KA7	TK0925	Phenylalanyl-tRNA synthase subunit β-chain	*Thermococcus kodakaraensis*	Antiphospho-Tyr antibody	Jeon *et al.* (2002)
Q5JH20	TK1404	Phosphomannomutase	*Thermococcus kodakaraensis*	Antiphospho-Tyr antibody	Jeon *et al.* (2002)
Q980S1	SSO0207	(*) Hexosephosphate mutase	*Sulfolobus solfataricus*	Phosphorylated on a Ser residue	Solow *et al.* (1998); Ray *et al.* (2005)
D4GYZ1	HVO_1562	β-Subunit of 20S proteasome	*Haloferax volcanii*	Phosphorylated on Ser^{129}	Humbard, Stevens and Maupin-Furlow (2006)
B0R4J9	CheA	Taxis sensor His kinase cheA	*Halobacterium salinarium*	Phosphorylated with [γ-^{32}P]ATP and Mg^{2+}	Rudolph and Oesterhelt (1996)
B0R4K1	CheY	Response regulator CheY	*Halobacterium salinarium*	Phosphorylated on Asp residue	Rudolph and Oesterhelt (1996)
D9PYR8	MTH1412	Cell division control protein 6 homolog 1	*Methanobacterium thermoautothrophicum*	Autophosphorylation with ^{32}P on Ser residue	Grabowski and Kelman (2001)
D9PU97	MTH1599	Cell division control protein 6 homolog 2	*Methanobacterium thermoautothrophicum*	Autophosphorylation with ^{32}P on Ser residue	Grabowski and Kelman (2001)
Q980N4	SSO0257	Cell division control protein 6 homolog 1	*Sulfolobus solfataricus*	Autophosphorylation with ^{32}P on Ser residue	De Felice *et al.* (2006)
Q8ZYK1	PAE0737	Cell division control protein 6	*Pyrobaculum aerophilum*	Phosphorylated on Ser residue	Grabowski and Kelman (2001)
O58655	PH0961	Translation initiation factor 2 subunit α	*Pyrococcus horikoshii*	Phosphorylated by hPKR from human	Tahara *et al.* (2004)
P95928	SSO2154	Zn-dependent aminopeptidase	*Sulfolobus solfataricus*	Phosphorylated on Ser or Thr residue	Condo *et al.* (1998)
–	–	(*) α-subunit of succinyl-CoA synthetase	*Sulfolobus solfataricus*	^{32}P incorporation	Ray *et al.* (2005)
–	–	Glycogen synthase	*Sulfolobus acidocaldarius*	^{32}P incorporation	Skorko (1989)
Q97U27	SSO3198	Gluconate dehydratase	*Sulfolobus solfataricus*	^{32}P-labeled, no enzyme activity after incubation with phosphatase	Kim and Lee (2005)
Q980A0	SSO0417	(*) Phosphoglycerate mutase	*Sulfolobus solfataricus*	Phosphorylation at Ser^{59}	Potters *et al.* (2003)
Q9HLV3	Ta0122	2-Keto-3deoxy-gluconate kinase	*Thermoplasma acidophilum*	No enzyme activity after incubation with phosphatase	Jung and Lee (2005)
Q4J9H3	ArnB	Regulation of archaellum expression	*Sulfolobus acidocaldarius*	Phosphorylated in WT, *saci_PP2A* and *saci_PTP* deletion mutant, Phosphorylated by two ePKs (Saci_1193 and Saci_1694)	Reimann *et al.* (2012, 2013)
Q4J9L2	ArnR1	Regulation of archaellum expression	*Sulfolobus acidocaldarius*	Phosphorylated in *saci_PTP* deletion mutant	Reimann *et al.* (2012, 2013)

(*) Possible phosphoenzyme intermediate.

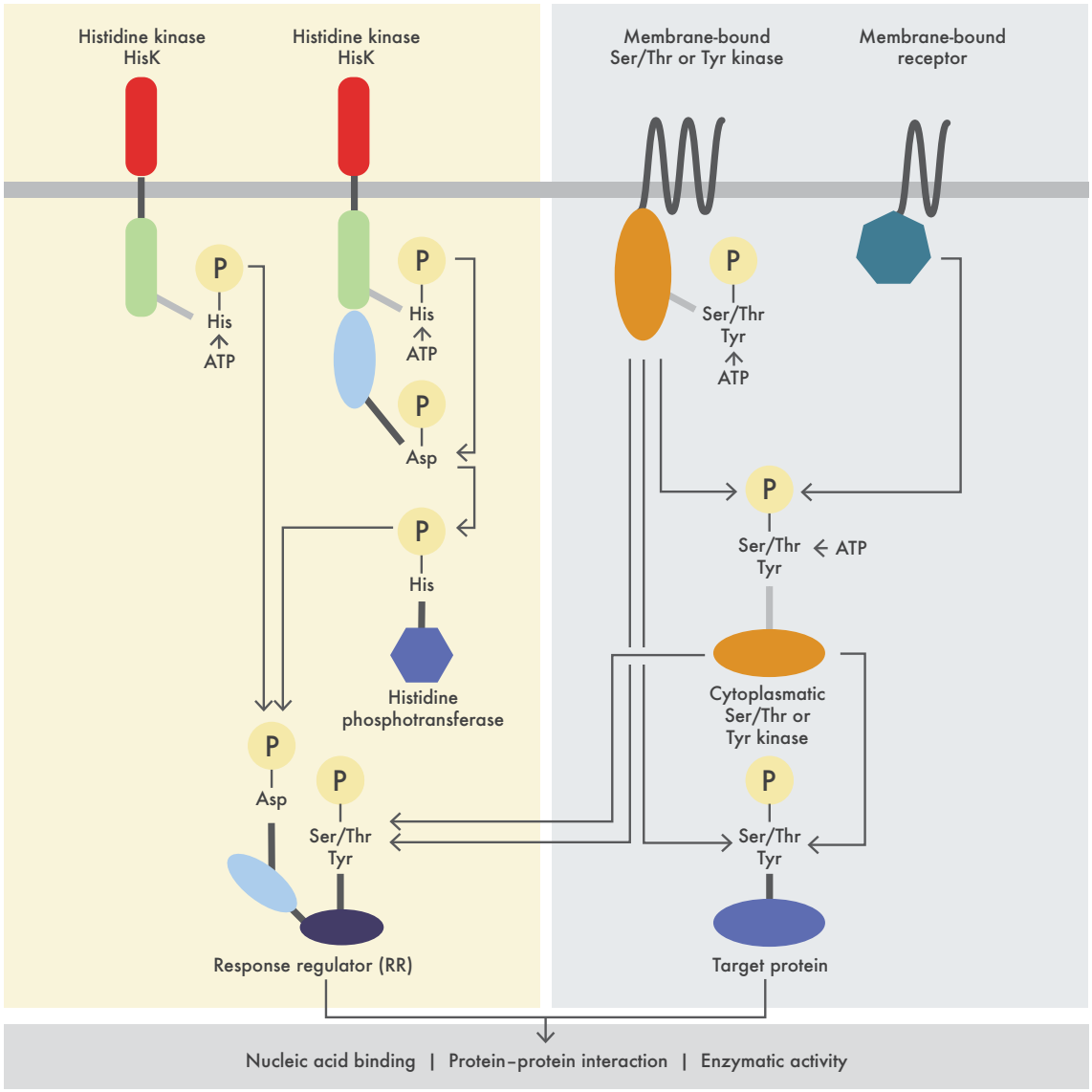

Figure 1. Signal transduction systems. In TCSs (left panel), the membrane-bound histidine kinase (HisK) receives an environmental stimulus via its external sensor domain (red), which leads to autophosphorylation of the cytoplasmic kinase domain (green) on a His residue. Subsequently, the signal is transduced to a cytoplasmic response regulator, which is phosphorylated on an Asp residue leading to a specific output. In contrast to TCSs, hybrid kinases are fused proteins bound to the membrane. They combine the sensor input domain of the HisK with the output domain of the RR. They can also transmit the phosphate moiety to a histidine phosphotransferase (pink), which in turn phosphorylates another RR leading to a specific output. Hanks-type kinases (right panel) are either membrane-bound or cytoplasmatic proteins (orange). Upon perception of a stimulus either directly via a membrane-bound receptor (light green), the kinases autophophosphorylate on a Ser/Thr or Tyr residue and transmit the signal to other kinases or directly to a target protein (purple) which leads to a specific output. Several Hanks-type kinases can be serially connected starting usually with a membrane-bound kinase (phosphorylation cascade).

cytosolic proteins (e.g. 97% of one-component regulators with HTH motif), whereas most of the sensor histidine kinases of TCSs (73%) were membrane bound. Thus, OCSs and TCSs were postulated to be involved in the detection of intracellular and extracellular signals, respectively (Ulrich, Koonin and Zhulin 2005). From an evolutionary point of view, it was suggested that OCSs are the predecessors of TCSs. This is based on three observations: (i) OCSs have a simpler design than TCSs, (ii) the domain architecture of one-component regulators is more versatile than that of TCSs and (iii) OCSs are more abundant in Prokaryotes compared to TCSs. Further on it was proposed that the last common ancestor of Archaea and Bacteria possessed OCSs, but lacked TCSs and that TCSs are an invention of Bacteria. The invention of TCSs occurred due to the insertion of the HisK domain and the receiver domain into OCSs (Ulrich, Koonin and Zhulin 2005). This theory would fit with the assumption that TCSs in Archaea were acquired via horizontal gene transfer from the Bacteria (Koretke *et al.* 2000).

Only a year after, the study by Ulrich, Koonin and Zhulin (2005), Galperin (2006, 2010) and Ashby (2006) analyzed the distribution, structure and diversity of genes encoding RRs and TCSs in Archaea, respectively. Galperin analyzed 4610 RRs encoded in 200 bacterial and archaeal genomes, and performed a detailed classification according to their domain structure. The study led to the identification of new output domains, which were, in some cases, assigned to established

protein domain families. Ashby analyzed 23 completely annotated and 3 partially annotated archaeal genomes (21 euryarchaeal and 2 crenarchaeal genomes) via BLASTP and gene category lists (Ashby 2006). This approach led to the identification of 489 putative TCS genes in 14 euryarchaeal genomes and none in crenarchaeal or nanoarchaeal genomes. Furthermore, no TCS genes were identified in the available Thermoplasmatales genomes (*Thermoplasma acidophilum*, *Tpl. volcanium* and *Picrophilus torridus*), *Methanocaldococcus jannaschii*, *Methanopyrus kandleri* and *Pyrococcus furiosus* all representing themoacidophilic/hyperthermophilic Euryarchaeota. Only in four euryarchaeal hyperthermophiles (*Archaeoglobus fulgidus* DSM4304 (OGT 83°C), *P. abyssi* GE5 (OGT 96°C), *P. horikoshii* OT3 (OGT 98°C), *Thermococcus kodakarensis* (OGT 85°C; Ng *et al.* 2000)) and one thermophile (*Methanothermobacter thermautotrophicus* Delta H (OGT 65°C)), TCS genes were identified. Members of the Thermococcales (*P. abyssi* GE5, *P. horikoshii* OT3, *T. kodakaraensis*) had only three TCS genes (0.146% of the genome), whereas *A. fulgidus* DSM4304 harbored 31 TCS genes (1.26% of genome) and the thermophilic archaeon *M. thermautotrophicus* 23 TCS genes (1.2% of the genome).

The absence of RR and TCS in Crenarchaota and Nanoarchaota, as well as some members of the Euryarchaeota (e.g. *Thermoplasmatales*), is discussed in regard to their genome complexity, unique lifestyles (e.g. symbiontic/parasitic for Nanoarchaota) as well as by their unchanging and unique habitat (e.g. thermoacidohiles) (Galperin 2004, 2005; Ashby 2006). In general, it is proposed that a more complex life style and habitat requires increased genome complexity (number of encoded genes) with more complex regulation at the gene level 'a higher IQ', reflected by an increased number of regulators.

In addition, another fact that might play a role is the adaptation to life at high temperature. The P-His and P-Asp bound are relatively high-energy bonds and therefore the thermodynamic and kinetic properties might be affected by temperature. Studies of the chemotaxis TCSs in the hyperthermophilic bacterium *Thermotoga maritima* (growth optimum at 80°C) revealed that the phosphorylation site of the RR CheY is only stable for 25 s at 50°C (150 s at 25°C) (Swanson, Sanna and Simon 1996). However, there are a few exceptions in Archaea, as outlined above, with *A. fulgidus* (Euryarchaota, optimal growth temperature at 83°C) and 31 TCS genes being the most impressive one. But so far none of the TCS in hyperthermophilic Archaea was analyzed.

Characterized TCSs in Archaea

Despite the fact that TCS genes have been identified in Euryarchaeota, their physiological role has not been studied in great detail (Bourret and Silversmith 2010). Studies so far only include TCS in halophilic (*Halobacterium salinarium*, *Haloferax volcanii*) or methanogenic (*Methanosaeta harundinacea*, *Methanosarcina barkeri*) Archaea. Alam and Oesterhelt (1984) observed that *H. salinarum* displayed chemotaxis. This process involved stochastic switching of the motility structure, the archaellum (formerly archaeal flagellum; Jarrell and Albers 2012; Albers and Jarrell 2015), but the proteins involved in this process were not known. However, it was assumed that homologs of the bacterial chemotactic signaling cascades such as CheA and CheY might be involved. Indeed, almost 10 years later Rudolph and Oesterhelt (1995) identified CheA, which is the HisK of the *H. salinarum* chemotactic system. A *cheA* deletion strain lost its chemotactic behavior, as reported for deletion strains of *Escherichia coli* and *Bacillus subtilis* (Oosawa, Mutoh and Simon 1988; Fuhrer and Ordal 1991). In the same year, Rudolph *et al.* (1995) also reported that the recombinant proteins CheA and CheY are active and form a TCS in *H. salinarium*. CheA was shown to autophosphorylate in presence of Mg^{2+} and [γ-^{32}P]ATP.

Since the experiments carried out by the Oesterhelt group, it has become clear that the archaeal motility structure is in contrast to the bacterial flagellum related to type IV pili and therefore its motor and protein composition is totally different (Jarrell and Albers 2012; Albers and Jarrell 2015). Therefore, it is surprising that the same chemotactic system is employed in Archaea and Bacteria to control the rotational direction of their motility structure. How this system has been adapted to achieve this will be topic of future studies.

TCS in Methanosarcinales—Recently, the regulation of methanogenesis by the FilI-FilRs TCS in *M. harundinacea* 6Ac was reported by Li *et al.* (2014). Before this study, they found that FilI (Mhar_0446) is responsible for the production of signaling molecules (carboxyl-acyl homoserine lactones) in *M. harundinacea*. It was demonstrated that carboxyl-acyl homoserine lactones control cell morphological transitions and influence the C-flux for CH_4 production and biomass formation (Zhu *et al.* 2012). The *M. harundinacea* 6Ac genome possesses three HisKs (Mhar_0446 (FilI), Mhar_0936, Mhar_1766) with the HisKA and HATPase domains, but only FilI exhibits the HisK characteristic two transmembrane domains. In addition, five putative RRs were identified (Mhar_0169, Mhar_0445 (FilR1), Mhar_0447 (FilR2), Mhar_1520, Mhar_2042), but only FilR1 and FilR2 possess an REC domain. The genome organization of the three genes (*filI*, *filR1*, *filR2*) suggested that *filI* and *filR2* form an operon, which was verified by RT-PCR. Phosphotransfer studies with FilI and both RRs confirmed that FilI phosphorylates FilR1 and FilR2; however, the addition of carboxyl-acyl homoserine lactone had no influence on the activity of FilI. Furthermore, ChiP-PCR studies revealed that the RR FilR1 binds to its own as well as to the filI-filR2 promoter, which is common for RRs from bacterial TCSs. Even more interesting was the finding that FilR1 binds to promoters of several genes/operons, encoding proteins essential for methanogenesis (*acs1* operon, *acs4* gene, *mtr* operon, *fwdCABD* operon and *omp* gene). This is the first study in *Methanosarcinales* indicating a positive regulation of methanogenesis by a TCS.

SER, THR AND TYR PHOSPHORYLATION IN ARCHAEA

Ser/Thr and Tyr phosphorylation in Eukaryotes and Archaea is carried out by specific eukaryotic PKs, called Hanks-type kinases. Today there are seven major clusters of Hanks-type PKs (also named ePKs) characterized. These are called the tyrosine kinase (TK) group; PK A, G and C families (AGC) group; the calcium and calmodulin-regulated PKs (CAMK) group; the tyrosine kinase-like (TKL) group; the cycline-dependent/mitogen-activated/glycogen synthase/cycline-dependent like PK (CMGC) group; homologs of yeast STE7, STE11 and STE20 PKs (STE) group; and the cell kinases (CK1) group (Manning *et al.* 2002; Taylor and Kornev 2011). All Hanks-type kinases share a conserved catalytic domain containing 12 subdomains (Fig. 2A). The Mg^{2+}-ATP molecule binds usually to the amino-terminal lobe (subdomain I–V) and the hinge region, whereas the substrate binds to the carboxy-terminal lobe (subdomain V–XI). The most important residues for catalytic function are as follows: the Lys (K) residue in subdomain II; the Asp (D) in subdomain VII, which is involved in the orientation and anchoring of the ATP; and the invariant Asp (D) in the subdomain VIB, which is likely to be the

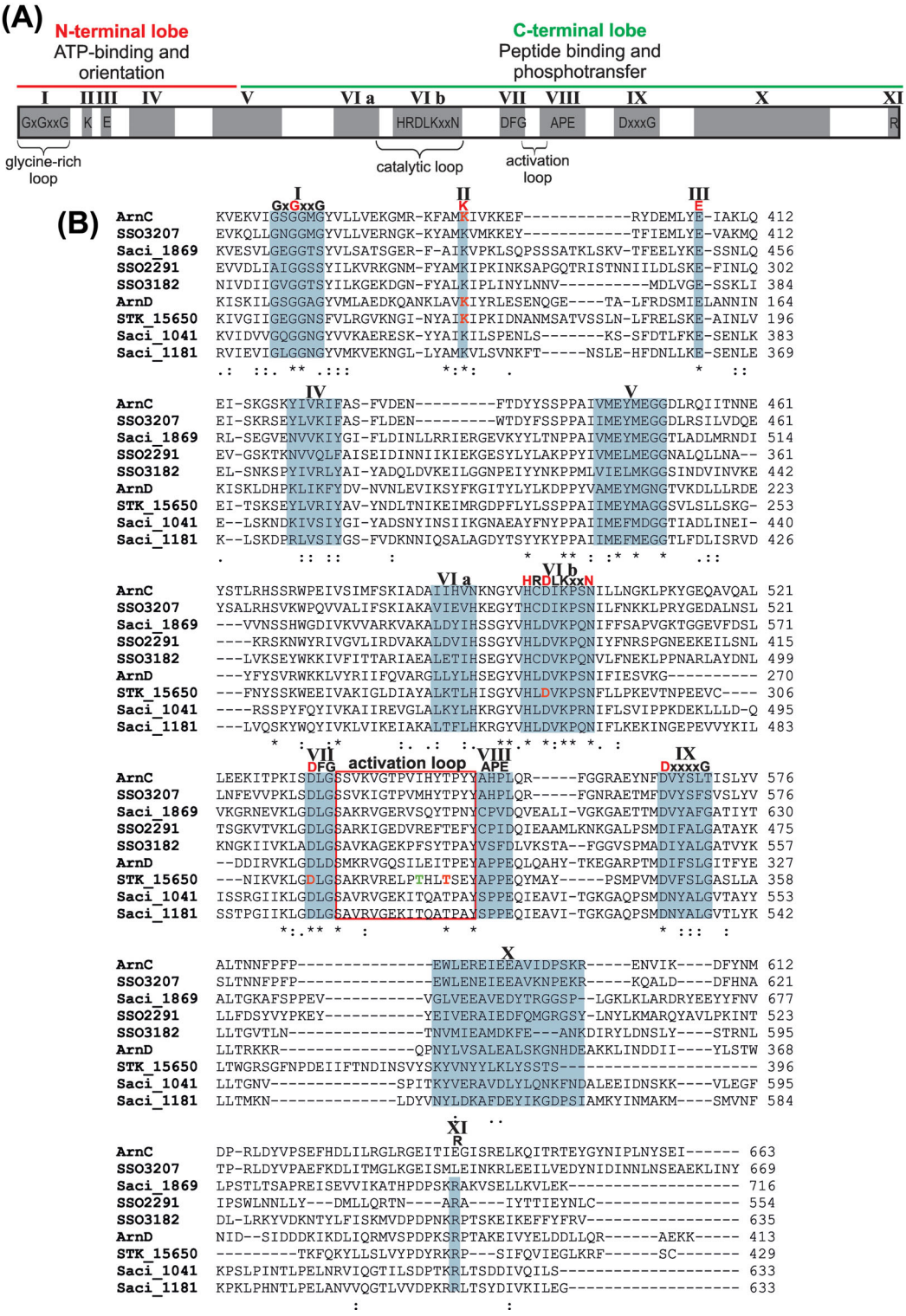

Figure 2. Conserved subdomains of Hanks-type PKs and alignment of *Sulfolobus* spp. Hanks-type PKs showing the 12 conserved subdomains. (**A**) The subdomains of Hanks-type Ser/Thr and Tyr kinases are represented with gray boxes and labeled with roman numbers. The boxes contain conserved amino acids important for function of the kinase domain. Locations of special regions within the domain that are of specific importance are labeled (e.g. catalytic loop) in black. (**B**) Alignment of Hanks-type kinases (eSTKs) of *S. soflataricus*, *S. acidocaldarius* and *S. tokodaii*. Highlighted in blue and labeled with roman numbers are the conserved Hanks-type PK subdomains. The consensus sequence of the motifs is depicted above the respective box. Subdomains without labeling have no conserved consensus sequence and seem to contribute mainly in supporting the structure of the kinase domain. Information about the location of these subdomains as well as important functional residues was obtained by comparing the available data for SSO3207 (Ray *et al.* 2015), modeled on the structural model of ArnC (Saci_1193) and ArnD (Saci_1694) as well as available data on the localization of secondary structure elements (Hanks and Hunter 1995; Kannan *et al.* 2007; Wang *et al.* 2010; Ray *et al.* 2015). Residues labeled in red and green have been identified in STK_15650 by combined mutational and *in vitro* studies and revealed to be essential for function or to decrease kinase activity, respectively (Wang *et al.* 2010). These residues are conserved in Hanks-type kinases (eSTKs) in *Sulfolobus* spp. The alignment was generated with Clustal Omega, * conserved residues, : strong similarity between residues,. weak similarity between residues. The N-terminal part of all sequences was trimmed and only the kinase domain is shown in the alignment.

catalytic base involved in the phosphotransfer reaction (Hanks 2003) (Fig. 2A).

Interestingly, in Bacteria, Hanks-type Ser/Thr PKs (eSTKs) are omnipresent, whereas Hanks-type Tyr PKs are rare and mainly members of the bacterial protein-tyrosine kinases (BY) family are found. The BY-kinases represent either single membrane proteins with a membrane and cytoplasmic part or are split into two proteins. The cytoplasmic part forms the catalytic site, which is characterized by three Walker A motifs (A, A' and B) usually found in ATPases and GTPases (Grangeasse, Nessler and Mijakovic 2012; Shi *et al.* 2014; Dworkin 2015). In addition to the two separate ways of phosphorylation (His/Asp and Ser/Thr/Tyr), more and more evidence for cross-reactions between eSTKs/ BY-kinases and transcriptional regulators emerges in Bacteria. Here, the eSTKs and BY-kinases phosphorylate transcription factors and RRs of TCS, which changes e.g. their DNA-binding behavior (Fig. 1) (Wright and Ulijasz 2014; Kalantari *et al.* 2015). Today, there are several different examples of Bacteria in which these cross-reactions were observed but no such interaction was described in Archaea, yet.

A detailed classification of ePKs in Archaea has not been performed so far. However, in 2014, it was shown that many Archaea contain at least one ePK and usually two phosphatases in their genome (Kennelly 2014). The most detailed studies regarding the presence of ePKs were performed with species of the Sulfolobales and so far only eSTKs were characterized (see Table 3). Blast searches (BlastP) with typical BY-kinases (PtkA, *Bacillus subtilis* and Wzc, *Escherichia coli*) reveal no obvious homologs in Archaea (Shi *et al.* 2014). Therefore, the PKs involved in Tyr phosphorylation remain to be elucidated in Archaea. As Crenarcharchaeota possess no TCSs, they solely rely on eSTKs for signal transduction and so they possess several of these kinases. For example, *Sulfolobus solfataricus* encodes 8 eSTKs and for *S. acidocaldarius* 11 are predicted based on arCOG functional annotation (Kennelly 2003; Esser *et al.* 2011). In contrast, euryarchaeal species were reported to have less eSTKs, but encode a variety of TCS (Ponting *et al.* 1999; Kennelly 2003).

eSTKs in Archaea

The only study concerning Ser, Thr and Tyr protein phosphorylation in methanogens was performed in *Methanosarcina barkeri* DSM 800 (Daas *et al.* 1996). The methyltransferase activation protein (MAP) was purified from the crude extract of *M. barkeri* and autophosphorylation was demonstrated in the presence of [γ-^{32}P]ATP. The phosphate bound was stable under acidic conditions, suggesting that the phosphorylation site is an *O*-phosphate linkage (Ser, Thr, Tyr residues). Incubation of the p-MAP with the corrinoid-containing methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT_1), resulted in activation of MT_1. The authors suggest that either p-MAP functions like a PK and phosphorylates MT_1 leading to an activated p-MT_1 or p-MAP does not covalently change MT_1, but affects the MT_1 structure and functions similarly to a chaperone. Since then, no studies regarding MAP from *M. barkeri* have been published, thus this question remains unsolved.

Most of the information available on archaeal eSTKs stems from studies in *Sulfolobus* spp. (see Table 3). Fifteen years ago, the Kennelly group started to characterize the PKs from the crenarchaeal model organism *S. solfataricus* (see Table 3). Since then, four ePKs and one non-canonical Hanks-type PK (atypical PK, aPK) were analyzed, applying various *in vitro* techniques in order to determine characteristics of the kinases, such as autophosphorylation behavior, specificity and ion preference (Table 1; for recent review, see Kennelly 2014). All of these ePKs performed phosphorylation on Ser/Thr (eSTKs), while preferring Mn^{2+} as ion for phosphorylation activity on non-native substrates such as casein, lysozyme or bovine serum albumin (BSA). Interestingly, SSO3184 (Sso-PK4) was proposed to be involved in the phosphorylation of the translation factor eIF2α (Ray *et al.* 2015). However, to date, information concerning the physiological role of these kinases in *S. solfataricus* remains scarce. *Sulfolobus acidocaldarius*, a closely related organism, contains 11 predicted PKs, of which at least 5 contain most or all of the conserved subdomains of eSTKs (compare Fig. 2B). Even though there is biochemical information on some kinases of *S. solfataricus* available and both organisms belong to the *Sulfolobales*, the information cannot be directly transferred between these two organisms because the homology between the kinases is often low and only the kinase domain is conserved but not the rest of the protein.

eSTKs in *Sulfolobus tokodaii*

Based on *in silico* analysis, eight putative eSTKs were identified in *S. tokodaii* strain 7 (STK_00364, STK_00686, STK_00778, STK_00810, STK_00899, ST1565, STK_19960 and STK_24400) (Wang *et al.* 2010). All of them contained the catalytic loop (DVKPSN), the DFG motif and the conserved residues K_{166}, D_{287} and D_{314} known from classical eukaryotic homologs (see Fig. 2B). The putative ePK STK_15650 was chosen for detailed characterization. In addition, Wang and coworkers performed a detailed analysis of forkhead-associated (FHA) domain-containing proteins in *S. tokodaii*. These proteins are known to be closely linked to pathways involving protein phosphorylation, since the FHA domain can recognize p-Thr epitopes on proteins. They are usually found in Eukaryotes as well as Bacteria and play an important role in the phosphorylation-dependent assembly of protein complexes (Durocher and Jackson 2002). Specific interaction of the kinase STK_15650 and the FHA domain-containing STK_00829 was demonstrated by *in vitro* analysis. The biochemical investigation of STK_15650 revealed that this PK is specific for Mn^{2+} as cofactor, and mutational studies revealed that the residues K_{166}, D_{287}, D_{314} and T_{329} (Fig. 2B, red labeled amino acids) are essential for the activity, resulting in an inactive enzyme when changed to A. The amino acids are located in subdomain II (invariant lysine), VIb (catalytic loop), VII (DFG motif) and finally, T_{329} is located inside the activation loop. Kinases often require phosphorylation of a specific residue within the activation loop in order to be active (Taylor and Radzio-Andzelm 1994; Nolen, Taylor and Ghosh 2004; Lochhead 2009). Interestingly, this residue is conserved in all other kinases presented in our alignment (Fig. 2), which suggests a similar mechanism of activation for all these kinases of *S. acidocaldarius* and *S. solfataricus*. However, there is currently no information available which proves this point from either one of these organisms. In contrast, the mutation of T_{326} to A in STK_15650 stimulated the PK activity, suggesting that T_{326} plays an important regulatory role in this ePK (Fig. 1, green labeled residue). This threonine is again conserved in two other kinases of *S. acidocaldarius* (Saci_1041 and Saci_1181), which implies that a similar mechanism for regulation like in *S. tokodaii* is present. However, this has not yet been studied.

STK_15650 was shown to phosphorylate the FHA domain-containing STK_00829, and the mutations of STK_15650 had the same effect on the phosphorylation of STK_00829 as on the autophosphorylation. Interestingly, Mg^{2+} had a positive effect on the phosphorylation of STK_00829. This was not observed for the autophosphorylation of the eSTK (STK_15650). To

Table 3. Characterized ePKs and aPKs from *Sulfolobus* spp.

ORF	Name	Membrane bound	Phosphorylated amino acid on substrate	Substrate	Preferred ion	Inhibitors	Additional information	Reference
Sso2291	Sso-PK1	Yes, 3 N-terminal TMDs	Ser/Thr	Casein, RCM-lysozyme (RCML), mixed histones/histone H4, specific peptide	Mg^{2+} (at 25°C) and Mn^{2+} (at 65°C)	PKI peptide, H7, ML-9	GTP, ADP and GDP could be used as cosubstrates, protein is glycosylated	Lower, Bischoff and Kennelly (2000); Lower and Kennelly (2002)
Sso0433	Sso-PK5	No	Ser	p53, casein, RCML	Mn^{2+}	Not tested	aPK, homolog of piD261/Bud32 kinase family, is activated by DNA, ADP-ribose and 5′-AMP	Haile and Kennelly (2011)
Sso0469	Sso-PK3	No	Ser (only casein on Ser/Thr)	Casein, BSA, Myelin basic protein (MBP), RCML	Mn^{2+}	Tamoxifen	No autophosphorylation detected	Lower, Potters and Kennelly (2004)
Sso2387	Sso-PK2	No	Ser	Mixed histones, RCML, BSA, casein	Mn^{2+}	Tamoxifen	-	Lower and Kennelly (2003)
Sso3182	Sso-PK4	Yes, 6 N-terminal TMDs	Ser/Thr	Casein, RCML, MBP, histones	Mn^{2+}	3′,5′-cAMP	Proposed homolog of archaeal eIF2α kinase, increased catalytic efficiency in presence of oxidized CoA	Ray *et al.* (2015)
STK15650	-	No	Not tested	STK0829	Mn^{2+}	Not tested	Interacts with and phosphorylates ST0829 *in vitro*	Wang *et al.* (2010)
Saci1193	ArnC	No	Ser/Thr	ArnA, ArnB	Mn^{2+}	Not tested	Phosphorylates two negative regulators of motility in *S. acidocaldarius*, ArnA and ArnB, *in vitro*	Reimann *et al.* (2012); Hoffmann *et al.* (unpublished)
Saci1694	ArnD	No	Ser/Thr	ArnB	Mn^{2+}	Not tested	Phosphorylates only ArnB *in vitro*	Reimann *et al.* (2012); Hoffmann *et al.* (unpublished)

conclude which residues in STK_00829 are important for interaction with the kinase STK_15650, bacterial two-hybrid experiments were performed with point mutations (R_{164}, S_{178}, T_{199} or N_{200} changed to A), revealing that all four residues seem to be essential for the interaction of both proteins. Subsequently, it was proposed that the FHA domain-containing protein bound in a phosphorylation-dependent manner to the *flaX* promoter (Duan and He 2011), which codes for a structural part of the archaellum (archaeal flagellum) assembly apparatus. However, *in vivo* confirmation has not been reported so far.

Signal transduction by eSTKs in *Sulfolobus acidocaldarius*: 'The archaellum regulatory network'

The findings obtained from *in vitro* studies in *S. tokodaii* were the first results reported on an archaeal signal transduction cascade involving eSTKs. However, the physiological function of this cascade was not elucidated until Reimann *et al.* (2012) showed that in *S. acidocaldarius* a regulatory system consisting of the homolog of ST0829, ArnA and various other proteins regulates expression of the motility structure, the archaellum. In *S. acidocaldarius*, an operon consisting of seven genes, which are expressed from two promoters encodes for the archaellum (Fig. 3) (Lassak *et al.* 2012). One promoter is located upstream of the archaellin encoding gene *flaB*, which is the filament protein of the archaellum. The second one is located upstream of the accessory protein *flaX* and has low constitutive activity. The main promoter for expression is the one upstream of *flaB* that was shown to be induced by starvation (Lassak *et al.* 2012). The expression of the archaellum has to be tightly regulated. Regulation is achieved on the transcriptional and posttranslational level by the archaellum regulatory network. Reimann *et al.* also showed that two regulatory proteins, ArnA and ArnB, as well as two kinases and one phosphatase are part of the complex archaellum regulatory network. ArnA and ArnB are located in an operon. ArnA contains a zinc-finger (ZnF) and FHA domain, while ArnB possesses a von Willebrand type A domain (vWA). Deletion of either one of the two repressors showed a hypermotile phenotype *in vivo* and protein levels of the archaellin FlaB were strongly enhanced compared to the WT strain (Reimann *et al.* 2012). Moreover, ArnA and ArnB interact tightly with each other in a phosphorylation-dependent manner. Here, the highly phosphorylated, flexible C-terminal part of ArnB interacts with the FHA domain of ArnA. Furthermore, ArnB binds to the *flaB* promoter forming a repression module with ArnA on the DNA (Hoffmann *et al.* in preparation). In addition to the negative regulators, there are two proteins flanking the archaellum operon, ArnR and ArnR1, identified as activators of archaellum expression (Lassak *et al.* 2013) (Fig. 3A). Both proteins are membrane-bound transcription factors and therefore considered to represent OCSs. They fulfill their function by binding to a specific target region upstream of the *flaB* promoter under starvation conditions. Even though both proteins consist of the same domains, they are only highly homologous with respect to their DNA-binding region (HTH domain), but differ from each other in the sensory region (HAMP domain and sensory domain close to the membrane anchor). This observation led to the conclusion that ArnR/R1 might be able to sense different signals. Interestingly, only *S. acidocaldarius* has two homologs, ArnR and ArnR1, in contrast to other *Sulfolobales*, which only encode *arnR* (Lassak *et al.* 2013).

Furthermore, the archaellum regulatory network includes at least two eSTKs, ArnC and ArnD. Both kinases belong to the eSTKs and are able to phosphorylate the negative regulators ArnA and ArnB *in vitro*. While the kinase ArnC is able to phosphorylate both ArnA and B, ArnD only phosphorylates ArnB (Reimann *et al.* 2012). Deletion studies of the kinases revealed that they have a different effect; while the *arnC* deletion resulted in reduced motility compared to the wt, *arnD*-deficient strains showed a hypermotile phenotype (for examples, see Fig. 3B). As a consequence, the assumption is that they fulfill different roles in the archaellum regulatory network. Since both kinases can phosphorylate the negative regulators of archaellum expression, it is assumed that they act on the repressor module (ArnA/B) (Hoffmann *et al.* submitted).

Finally, yet importantly, the phosphatase Saci_PP2A is another factor in this regulatory cascade. Reimann *et al.* (2013) showed that deletion of the respective gene resulted in a hypermotile phenotype *in vivo* (see also below Saci phosphatases). Furthermore, transcriptome analysis as well as qRT-PCR and western blots revealed that a Δ*saci_pp2A* strain mimicked the starvation phenotype of *S. acidocaldarius*. Components of the archaellum were strongly expressed and high FlaB protein levels could be detected in the deletion strain, as is usually found under starvation conditions (Reimann *et al.* 2013). Interestingly, the archaellum regulators ArnR1 and ArnB were also observed to be phosphorylated *in vivo* in a phosphoproteome study performed with the *saci_pp2a* and *saci_ptp* deletion mutants.

To date, the archaellum regulatory network represents the first archaeal signal transduction cascade involving eSTKs and PP investigated so far, including regulation at the transcriptional as well as post-translational level.

NON-CANONICAL HANKS-TYPE PKs (aPKs)

Apart from ePKs the non-canonical Hanks-type PKs (aPKs) of the ABC1, right open reading frame (RIO), piD261, AQ578 and Pkn2 families were introduced by Leonard, Aravind and Koonin (1998). Their bioinformatics study, based on 12 bacterial, 4 archaeal and 2 eukaryotic genomes, revealed that the aPKs are distant members of the ePK superfamily and that with exception of the Pkn2 family, all aPK families are found within the Archaea. Based on the structural comparison, it was evident that aPks comprise the typical ePK subdomains I, II, VIb and VII with the conserved residues (Fig. 4). Furthermore, the DFG triplet, which chelates Mg^{2+}/Mn^{2+} ions, is well conserved in the identified piD261/Bud32 and ABC1 members, whereas this triplet differs in the AQ578 and RIO family members. However, the Asp residue in the DFG triplet is conserved in all of them. Interestingly, subdomain VIII (APE consensus sequence), which is usually phosphorylated in ePKs and important for ePK activation, is absent in aPK members. Nevertheless, for the *Saccharomyces cerevisiae* piD261/Bud32 homolog (YGR262C), which lacks the APE motif, activity was demonstrated suggesting that this motif is not strictly essential for catalytic activity of aPKs (Stocchetto *et al.* 1997).

Archaeal piD261/Bud32 family

In yeast, piD261/Bud32 is, together with the kinase-associated endopeptidase 1 (Kae1), part of the KEOPS (kinase, peptidase and other proteins of small size) protein complex also known under the name EKC (endopeptidase-like and kinase associated to transcribed chromatin) protein complex that is essential for telomere elongation and transcription of essential genes. This complex is composed of four or five subunits (i.e. Pcc1p, Gon7p (unique to fungi), Cgi121p, piD261/Bud32 and Kae1) (Downey

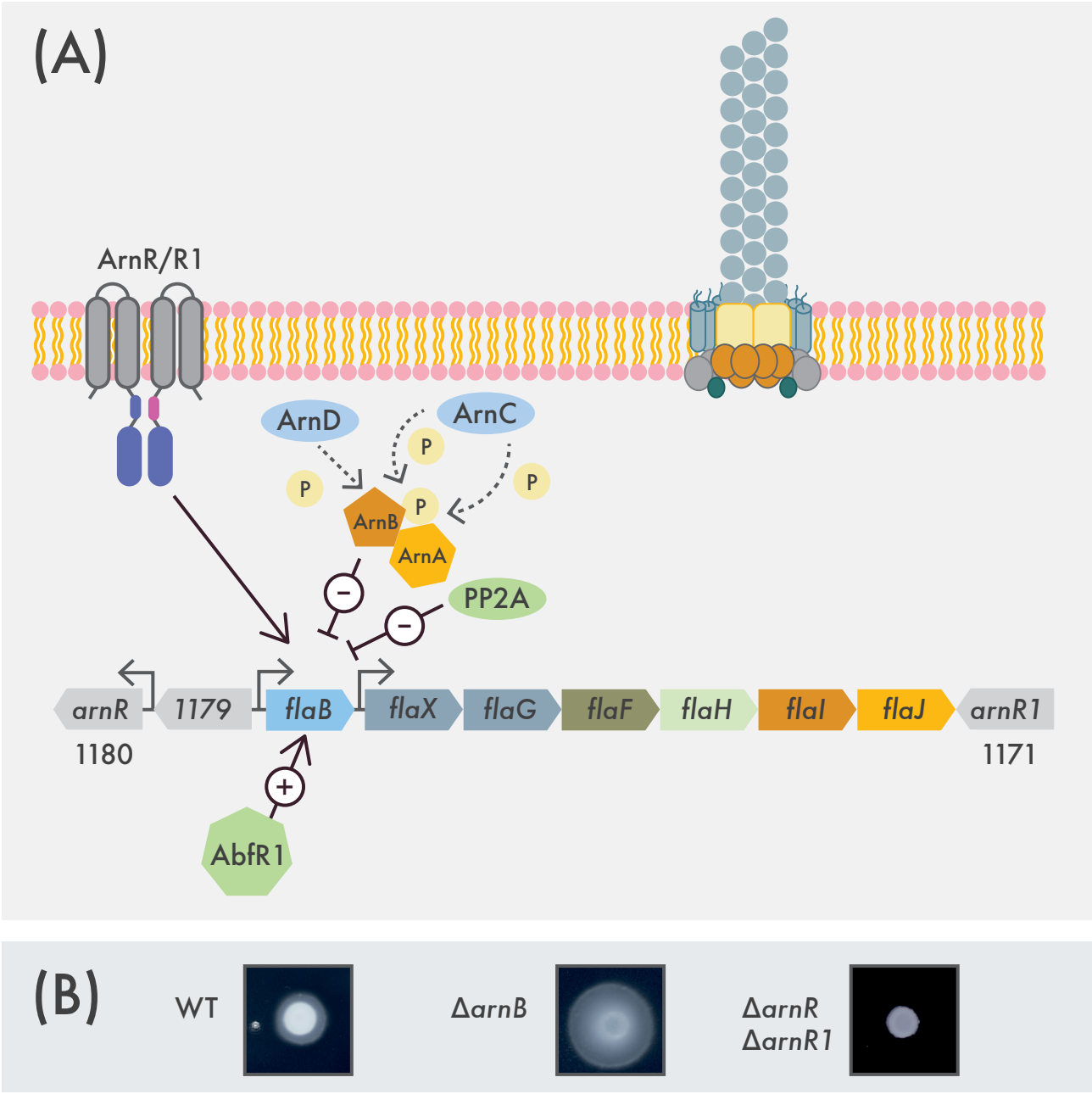

Figure 3. The current view of the archaellum regulatory network. (**A**) The archaellum locus flaBXGFHIJ encodes the archaellum, a rotating, type IV pilus-like structure which functions as the motility structure of *S. acidocaldarius*. The encoding genes and the respective proteins in the archaellum are depicted in the same colors. The locus is under the control of two promotors, the main flaB promotor and a second weak flaX promotor (transcription start sites are indicated by arrows). The locus is flanked by the genes encoding the one-component membrane-bound transcription factors ArnR and ArnR1, as well as Saci_1179 (protein of unknown function). In addition to ArnR and ArnR1, the biofilm regulator AbfR1 (Saci_0446) has been identified as positive regulator of the flaB promoter (depicted by (+) arrow) (Lassak *et al.* 2013; Orell *et al.* 2013). On the other hand, the two repressors, ArnA (Saci_1210) and ArnB (Saci_1211), are negatively regulating flaB expression (depicted by (–) arrows). In addition, two eSTKs, ArnC and ArnD, can phosphorylate the repressors ArnA and ArnB. Here, ArnD seems to be involved in repression, too, since its deletion results in hypermotility. Furthermore, the phosphatase PP2A seems to play an important function in the negative control of the motility operon, as well, since a pp2a deletion mutant exhibits a hypermotile phenotype, too. However, the specific target of the PP2A that relays the signal is so far unknown (adjusted from Albers and Jarrell 2015). (**B**) Example of swimming motility assays of the *S. acidocaldarius* MW001 (parental strain), and the ΔarnB as well as ΔarnR/arnR1 deletion mutants. The deletion of the negative regulator ArnB results in a hypermotile phenotype and of the positive regulators ArnR and ArnR1 in a non-motile phenotype.

et al. 2006; Gavin *et al.* 2006; Kisseleva-Romanova *et al.* 2006). Strikingly, whereas Kae1 is found in all three domains of life, orthologs of the piD261/Bud32 aPKs are only present in Eukaryotes and Archaea (Lopreiato *et al.* 2004). The diversity of analyzed archaeal piD261/Bud32 members is much greater than that of archaeal ePKs. So far three piD261/Bud32 aPKs from the Euryarchaeota (*Haloferax volcanii*, *Pyrococcus abyssi* and *Methanocaldococcus jannaschii*) and one enzyme from the Crenarchaeota (*Sulfolobus solfataricus*) were characterized (Hecker *et al.* 2008, 2009; Haile and Kennelly 2011; Naor *et al.* 2012). Interestingly, in nearly all archaeal genomes, the two genes are found in juxtaposition with each other and in some euryarchaeal

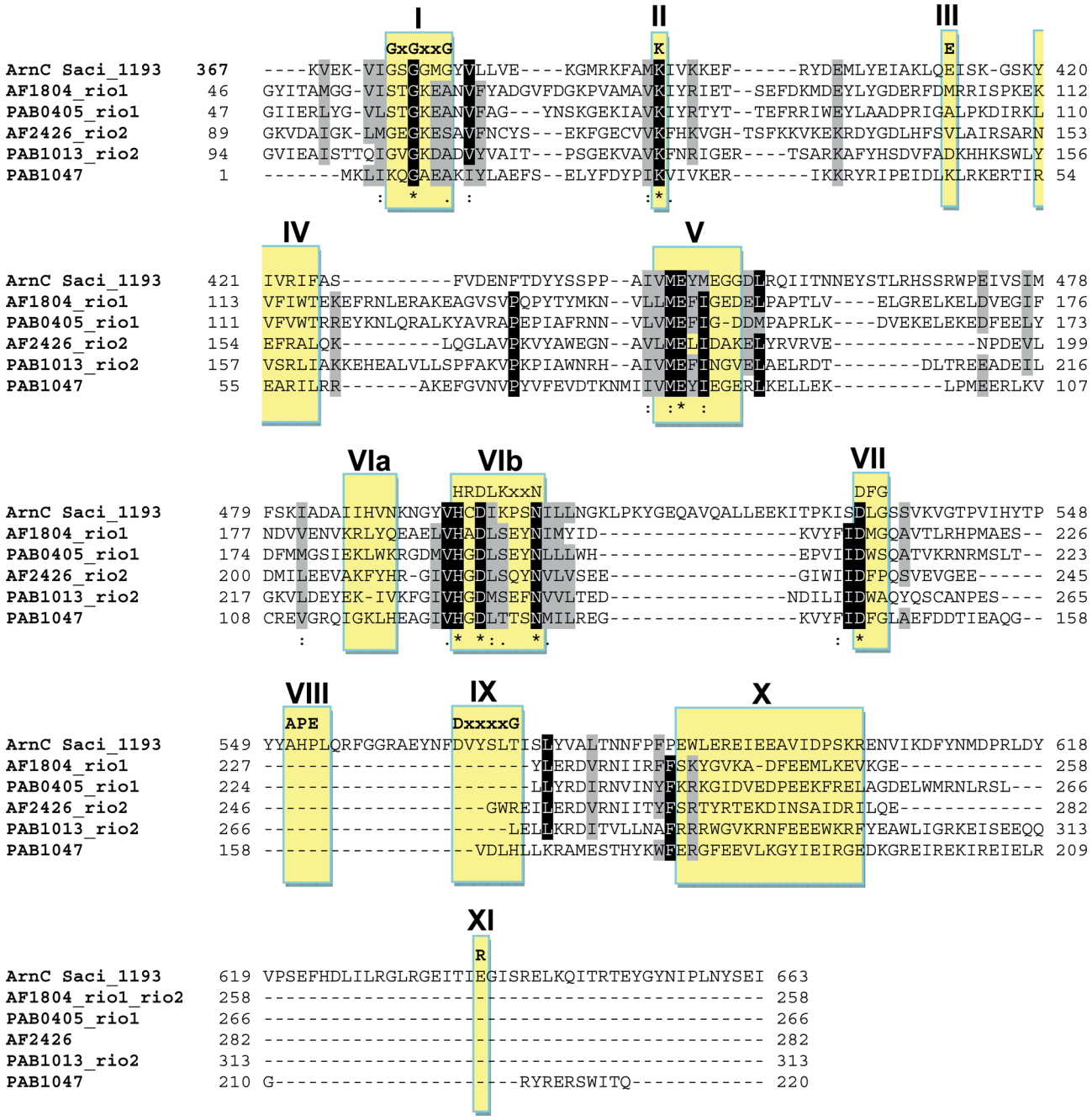

Figure 4. Amino acid sequence comparison of archaeal canonical Hanks-type PKs (eSTKs) and non-canonical Hanks-type PKs (aPKs) of the Rio1, Rio2 and piD261/Bud32 family. In the alignment, the eSTPKs from *S. acidocladarius*, *S. solfataricus* and *S. tokodaii* were included (as shown in Fig. 1), but for size reduction only ArnC (Saci_1193) is depicted. In addition, Rio1 and Rio2 kinases from *A. fulgidus* and *P. abyssi* as well as the piD261/Bud32 family member from *P. abyssi* were included. The conserved subdomains of Hanks-type Ser/Thr and Tyr kinases are indicated by yellow boxes and conserved residues are indicated above the sequence (as shown in Fig. 1). The alignment was generated with Clustal Omega, * conserved residues (black shading), : strong similarity between residues (gray shading),. weak similarity between residues.

genomes both genes are even fused (Marcotte *et al.* 1999). The structure of the piD261/Bud32-Kae1 fusion protein from the euryarchaeon *M. jannaschii* (MJ1130) has been solved and thereby it was confirmed that the N-terminal part (residues 1–323) corresponds to Kae1, followed by a linker region (residues 324–340) and piD261/Bud32 (residues 341–532) (Hecker *et al.* 2008).

Notably, the structure of *Mj*-piD261/Bud32 also resembles the available structures of aPKs of the RIO kinases family from *Archaeoglobus fulgidus* (LaRonde-LeBlanc and Wlodawer 2005; LaRonde-LeBlanc *et al.* 2005a,b), despite the rather low-sequence identity, and represents a shortened version of classical RIO kinases (Fig. 5). In the structure of the piD261/Bud32 domain, no nucleotide binding in the ATP-binding pocket was observed (inactive conformation), whereas the *Mj*-Kae1 domain had the nucleotide clearly bound. This finding supports the previously observed negative regulation of the piD261/Bud32 PK activity

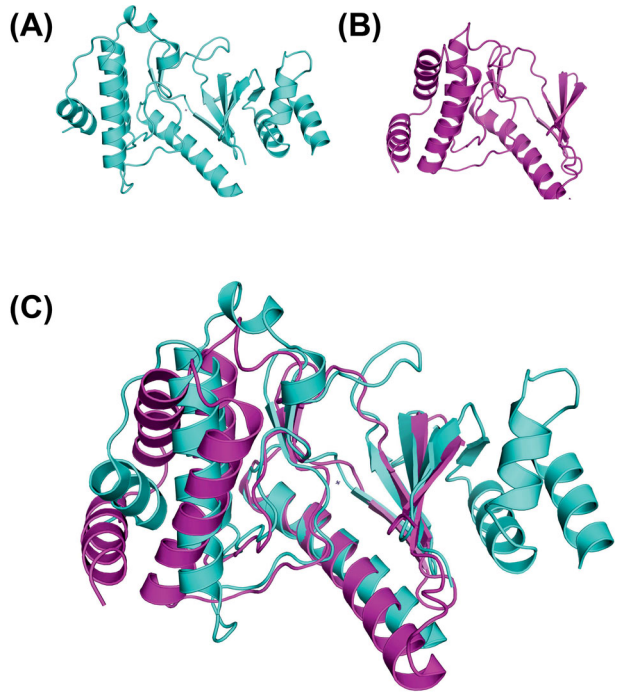

Figure 5. Structural comparison of archaeal non-canonical Hanks-type PKs (aPKs) of the Rio1 and piD261/Bud32 family. The structure of the Rio1 kinase from *A. fulgidus* (LaRonde-LeBlanc *et al.* 2005b) (**A**) and the piD261/Bud32 family member from *M. jannaschii* (C-terminal domain of the Kae1-Bud32 fusion protein) (Hecker *et al.* 2008) (**B**) as well as an overlay of both structures (**C**) is depicted.

by Kae1 (Hecker *et al.* 2008) and suggests that Kae1 inhibits the piD261/Bud32 aPk by keeping the ATP-binding site in an inactive confirmation (Hecker *et al.* 2009). Another investigated archaeal piD261/Bud32 member is SSO0433 from the crenarchaeon *S. solfataricus* P2, which was characterized in some detail (Haile and Kennelly 2011, see also Table 3).

In the hyperthermophilic euryarchaeon *P. abyssi*, three putative aPKs (PAB0405, PAB1013 and PAB1047) with good similarity to RIO kinases were identified (Hecker *et al.* 2009). Sequence comparisons revealed that PAB1047 is a member of the piD261/Bud32 family, whereas PAB0405 is a member of the Rio1 family and PAB1013 of the Rio 2 family. All three aPKs were able to phosphorylate myelin basic protein (MBP) *in vitro* in the presence of Mg^{2+} and for PAB1013 and PAB1047 an increase in activity was observed in the presence of Mn^{2+}. As shown for yeast and *M. jannaschii*, *Pab*-piD261/Bud32 interacts with Kae1 and the *P. abyssi* Kae1 ortholog (PAB1159) exhibits very low ATPase activity, leading to an autophosphorylation of the enzyme (Hecker *et al.* 2009). Furthermore, binding of single- and double-stranded DNA was shown for *Pab*-Kae1, which was inhibited by ATP. Apart from the DNA-binding activity, class I apurinic endonuclease activity was demonstrated using depurinated DNA as a template and it was thus suggested to rename Kae1 from kinase-associated endopeptidase 1 to kinase-associated endonuclease 1 (Hecker *et al.* 2009). In a recent study, the function of the *Pab*-KEOPS/EKC complex in the biosynthesis of N^6-threonylcarbamoyl adenosine (t^6A) modified tRNAs was demonstrated (Perrochia *et al.* 2013). The t^6A tRNA modification (at position 37, 3′ of the anti-codon, in all tRNAs that pair with ANN codons) is found in almost all organisms (except endosymbionts with highly reduced genomes) and plays an essential role for cell growth and translation fidelity. The authors propose that piD261/Bud32 functions as P-loop ATPase rather than as bona fide PKs, at least in conjunction with Kae1 in the KEOPS/EKC complex or in species that form single fusion proteins such as *M. jannaschii* or *Haloferax* spp. (Perrochia *et al.* 2013). Whether the piD261/Bud32 aPK might function as PK if it is not bound to Kae1 remains to be elucidated. ATPase function is also proposed for Rio 2 kinases.

Archaeal RIO kinases

Archaeal RIO kinases have been the focus of several studies in the last few years. RIO kinases were originally identified in Archaea and Eukaryotes and a common origin with an ancestral RIO gene has been predicted (Leonard, Aravind and Koonin 1998). Until now, four different subfamilies of RIO kinases were identified: Rio 1, Rio 2, Rio 3 and Rio B kinases (LaRonde-LeBlanc and Wlodawer 2005). Phylogenetic analysis confirmed the distribution of RIO kinases in all three domains of life and proposed the presence of a combination of one Rio 1 kinase and one Rio 2 kinase in less complex species (i.e. Prokaryotes and single cellular Eukaryotes), whereas multicellular Eukaryotes including humans also possess Rio 3 kinases (Manning *et al.* 2002).

In general, RIO kinases are regarded as a trimmed version of canonical ePKs that lack the subdomains VIII, X and XI (involved in substrate binding) (Laronde-Leblanc and Wlodawer 2004) (Fig. 6). RIO kinases possess an insertion of 18–23 amino acids between the αC and β3 (flexible loop), which is absent in ePKs. In general, Rio 1 and Rio 2 kinases are very similar regarding their overall fold, despite the N-terminal domain of the Rio 2 family comprising a winged HTH (wHTH) domain, which is absent in all other RIO families. Members of the different RIO families can be distinguished by their specific P-loop (interaction and orientation of the ATP triphosphate moiety) and DFG-loop (metal-ion binding and positioning) sequence (LaRonde-LeBlanc *et al.* 2005a,b). In contrast to Prokaryotes, the role of RIO kinases in Eukaryotes, where they play a major role in ribosome synthesis, is well established (for recent review, see Laronde 2014). While Rio1 fulfills an essential role in chromosome maintenance and cell cycle progression, both Rio kinases are involved in 18s rRNA progression. Therefore, deletion of either one of the two RIO kinases in yeast results in cell death (Vanrobays *et al.* 2001, 2003; Angermayr, Roidl and Bandlow 2002; Giaever *et al.* 2002; Geerlings *et al.* 2003; Schäfer *et al.* 2003). In humans, RIO kinase activity was shown to be essential in small ribosomal subunit (40S) biogenesis, and recent studies indicate that Rio1, 2 and 3 function as ATPase rather than PK in the maturation of the pre-40S particle (Kiburu *et al.* 2014; Laronde 2014). The function of RIO B is still unclear.

A major breakthrough was achieved based on the crystallization of the archaeal Rio 1 and Rio 2 kinases from *A. fulgidus* (Laronde-Leblanc and Wlodawer 2004; LaRonde-LeBlanc *et al.* 2005a). For both Rio 1 and Rio 2 kinase of *A. fulgidus*, autophosphorylation in the presence of labeled ATP was demonstrated and phosphorylation of common artificial kinase substrates was shown (Laronde-Leblanc and Wlodawer 2004; LaRonde-LeBlanc *et al.* 2005a; Hecker *et al.* 2009). Unusually, in this case, the observation that Af-Rio2 was autophosphorylated in the absence of Me^{2+}-ions and Ser_{128} was identified as the autophosphorylation site (Laronde-Leblanc and Wlodawer 2004; Hecker *et al.* 2009).

Bioinformatic analysis based on genome neighborhood analysis and sequence comparisons revealed that in all archaeal sequenced genomes (121 in August 2012) members of the RIO family are present (Esser and Siebers 2013). All archaeal species analyzed contain at least one (RIO 1 or RIO 2 kinase) but typically two

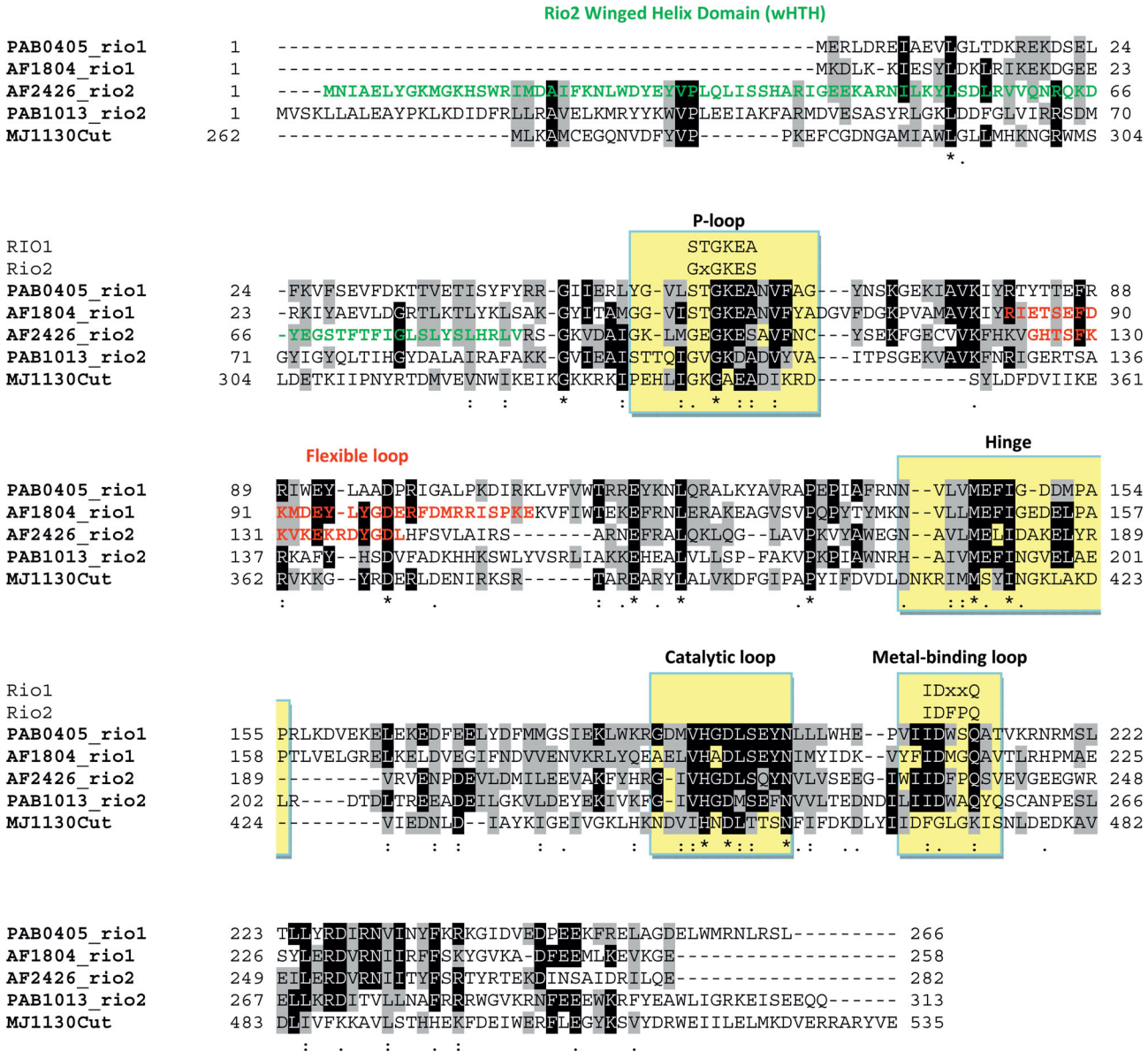

Figure 6. Amino acid sequence comparison of archaeal non-canonical Hanks-type PKs (aPKs) of the Rio1, Rio2 and piD261/Bud32 family. The Rio1 and Rio2 kinases from *A. fulgidus* and *P. abyssi* as well as the piD261/Bud32 family member from *M. jannaschii* (C-terminal domain of the Kae1-Bud32 fusion protein) were included. The two *A. fulgidus* Rio kinases and the *M. jannaschii* Bud32 are well studied and the crystal structures are available (Laronde-LeBlanc and Wlodawer 2004, 2005; LaRonde-LeBlanc *et al.* 2005a; Hecker *et al.* 2008, 2009). The typical Rio kinase domain features (yellow boxes and flexible loop (red), identified sequence features specific for the Rio1 and Rio2 family (sequences shown above the alignment) as well as the Rio2 specific wHTH domain with possible DNA-binding function (green, *A. fulgidus* Rio2)) are depicted (Laronde-LeBlanc and Wlodawer 2004, 2005; LaRonde-LeBlanc *et al.* 2005b). The alignment was generated with Clustal Omega, * conserved residues (black shading), : strong similarity between residues (gray shading),. weak similarity between residues.

copies of either RIO 1 and RIO 2, Rio B and Rio 2 kinase, or in a few cases two Rio 2 kinases. Interestingly, in all archaeal genomes, a conserved clustering of RIO genes together with genes encoding KH domain proteins (human KH (K homology) domain protein hnRNP (heterogeneous nuclear ribonucleoprotein), aeIF-1A (archaeal translation initiation factor-1A), DUF (domain of unknown function) 460, DEAD/DEAH-box helicases, snRNP (small nuclear ribonucleoproteins) or CbiA (cobyrinic acid a,c-diamide synthase)) were observed. In addition, members of the *Sulfolobaceae* seem to be special in this regard. In those species, a strictly conserved genome neighborhood of nine genes was identified, which are all related to transcription and translation regulation (Esser and Siebers 2013).

The only functional role in Archaea has been reported for the Rio 1 kinase from *H. volcanii*, which is capable of phosphorylating the α1 protein of the proteasome 20S core particle *in vitro* (Humbard *et al.* 2010). *In vivo* analyses after mutation of the phosphorylation sites of α1 revealed an important role of proteasome phosphorylation for pigmentation and cell viability. Mutation studies of Ser58Ala, Thr147Ala or Thr158Ala reduced the kinase activity of *Hvo*-Rio1 significantly. In addition, there is some structural evidence from comparison to their eukaryotic counterparts that suggests a similar role of archaeal RIO kinases in ribosome biogenesis with ATPase, rather than a kinase function (Laronde 2014). Therefore, the *in vivo* function of RIO kinases in Archaea is very unclear and requires further study.

PPs IN ARCHAEA

The counterparts of PKs are the PPs, which remove covalently linked phosphate groups. Overall, three families of PPs are known: (1) phosphatases active on pSer and/or pThr, (2) the ones which dephosphorylate pTyr (PTP) and (3) aspirate-based phosphatases such as the TFIIF-associated component of RNA polymerase II carboxy-terminal domain phosphatase (FCP) and the small carboxy-terminal domain phosphatases (SCP) (Shi 2009). Investigations in Eukaryotes also revealed the presence of atypical PPs, which were, so far, not detected in Prokaryotes (Sadatomi *et al.* 2013).

Ser/Thr PPs can be further classified into two subfamilies, the protein Ser/Thr phosphatases (PPPs) and the Mg^{2+}- or Mn^{2+}-dependent protein phosphatases (PPM). In Archaea, both subfamilies are present. PPPs are usually responsible for the dephosphorylation of pSer/pThr in Eukaryotes and Prokaryotes, whereas the PPMs are more common in Bacteria (Barford 1996; Cohen 1997; Kennelly 2002, 2003). A great difference between members of the PPM and PPP subfamilies is that PPPs usually possess different subunits. The heterodimeric PPPs consist of the catalytic subunit and a scaffold subunit, whereas the heterotrimeric PPPs have an additional regulatory subunit. The interplay between the different subunits determines substrate specificity (reviewed in Virshup and Shenolikar 2009). In contrast, PPMs are monomeric and contain sequence motifs and additional domains, which might mimic the presence of regulatory subunits (Lower, Bischoff and Kennelly 2000; Virshup and Shenolikar 2009). The PTP family can be subdivided into three groups, the PTPs, which are specific for pTyr dephosphorylation; the dual-specific PTPs, which can dephosphorylate pSer/pThr as well as pTyr; and the low-molecular-weight PTPs (<18 kDa). They all share one common amino acid motif, CX_5R (Shi, Potts and Kennelly 1998).

Since the discovery of protein phosphorylation in Archaea, four PPPs (*S. solfataricus* (Kennelly *et al.* 1993; Leng *et al.* 1995), *Methanosarcina thermophila* (Oxenrider *et al.* 1993; Solow, Young and Kennelly 1997), *Pyrodictium abyssi* (Mai *et al.* 1998) and *Haloferax volcanii* (Oxenrider and Kennelly 1993)), one PPM (*Tpl. volcanium* (Dahche *et al.* 2009)) and three PTPs (*S. solfataricus* (Chu and Wang 2007), *S. acidocaldarius* (Reimann *et al.* 2013) and *T. kodakaraensis* (Jeon *et al.* 2002)) were analyzed (for a review, see Kennelly 2014).

Archaeal PPPs

Archaeal PPs of the PPP group resemble their eukaryotic orthologs regarding their amino acid sequences. However, to date, only homologs of the catalytic subunit have been detected in Archaea and the biochemical properties seem to be different, especially regarding their inhibition behavior. The first archaeal PP which was characterized was the enzyme from the euryarchaeon *H. volcanii* (Oxenrider and Kennelly 1993). PP activity was demonstrated in soluble extracts with pSer/pThr substrates (i.e. casein, mixed histones and phosphorylase A), whereas no activity could be detected with pTyr substrates (i.e. reduced, carboxymethylated or maleylated lysozyme). Activity was stimulated by Mn^{2+} and Cd^{2+} ions, but inhibited by NaF, Zn^{2+}, vanadate, molybdate, inorganic phosphate/pyrophosphate, *p*-nitrophenyl phosphate and diethylpyrocarbonate. The addition of more specific PP inhibitors like okadaic acid and microcystin-LR, which are common inhibitors of eukaryal PP1/2A/2B PPs, had no influence on the *Hvo*-PP. At the same time, similar studies were performed in *S. solfataricus* P1. The native PPP from *S. solfataricus* P1 was isolated from the soluble fraction and was shown to dephosphorylate ^{32}P-labeled target proteins like casein (Kennelly *et al.* 1993). No PPP activity was detected with pTyr-labeled substrates, revealing that the enzyme is a protein Ser/Thr phosphatase. Furthermore, the *Sso*-PP revealed a strong Me^{2+}-ion dependency, using Mn^{2+}, Ni^{2+}, Co^{2+} and Mg^{2+} as cofactors. A few years later, the amino acid sequence of the *Sso*-PP was identified and the highest similarities towards eukaryal members of the PP1/2A/2B super family were observed (Leng *et al.* 1995). However, whereas eukaryal PP1/2A/2B members are sensitive towards low amounts of okadaic acid, calyculin A and microcystin-LR (Cohen 1991), no inhibition was observed for the *S. solfataricus* ortholog (Kennelly *et al.* 1993). In addition, *Sso*-PP1 was shown to be active as a monomer, showing high similarity towards the known catalytic subunits from the eukaryal heterooligomeric PPPs.

Only recently, the PP from *S. acidocaldarius* DSM639 was investigated, revealing similar biochemical properties (Reimann *et al.* 2013). Based on the sequence alone, the *S. acidocaldarius* ortholog can be assigned as a member of the PP2A branch of the PPP super family, which usually requires metal ions for activity and are involved in a variety of cellular processes like cell cycle control and cell mobility (reviewed in Shi 2009). The *Saci*-PP2A also requires Me^{2+} ions for activity, showing highest activity with Cu^{2+}. Activity was only observed with a pThr peptide (RRA(pT)VA), whereas no activity was detected with a pTyr peptide (TEVGKRI(pY)RLVGDKN), which was identified during the phosphoproteome study conducted in *S. acidocaldarius* (see below; Reimann *et al.* 2013). However, the *Saci*-PP2A was strongly inhibited by okadaic acid (56% at 10 nM okadaic acid), which showed no inhibitory effect on the *Sso*-PP1 enzyme. Furthermore, a function in archaellum regulation was suggested. The deletion of *pp2a* led to hypermotile cells and, therefore, it is likely that PP2A plays a role in repression of archaellum expression (see also above, Reimann *et al.* 2013).

The analysis of soluble extracts from *M. thermophila* TM-1 (Oxenrider *et al.* 1993; Solow, Young and Kennelly 1997) revealed, in a similar way to the *S. solfataricus* enzyme, PP activity with ^{32}P-casein in the presence of Mn^{2+}, Ni^{2+} and Co^{2+}. The addition of Cd^{2+}, Cu^{2+} or Zn^{2+} inhibited the PP activity completely. Activity could be demonstrated with pSer/pThr substrates, whereas no activity was detected with pTyr substrates, suggesting that the *Mth*-PP is also specific for pSer and pThr. Similar to the results for *Saci*-PP2A, but in contrast to *Sso*-PP1, *Mth*-PP was also very sensitive towards the specific PP1/2A/2B inhibitors okadaic acid, microcystin-LR and calyculin A (μM range). Four years later, the sequence of the *Mth*-PP was identified, revealing that this PP belongs to the PP1-arch2 branch (Solow, Young and Kennelly 1997). *Mth*-PP1 was heterologously expressed in *Escherichia coli* and the previously reported activity from soluble extracts was confirmed, demonstrating that PP1/2A/2B PPs are present in both major phyla of the Archaea, Crenarchaeota and Euryarchaeota.

The PP from the euryarchaeon *Pyd. abyssi* shows a high similarity to the enzymes of *S. solfataricus* and *M. thermophila* (Mai *et al.* 1998) and belongs to the PP1 branch within the PP1/2A/2B super family. Similar to the previous studies, *Pdab*-PP1 requires Me^{2+} ions and the highest activity was observed with Mn^{2+}, Co^{2+} and Ni^{2+}, decreased activity with Mg^{2+} and no activity at all in presence of Ca^{2+}. *Pdab*-PP1 was also inhibited by Cu^{2+}, Zn^{2+}, Fe^{2+}, NaF, NaK tartarte, DEPC and okadaic acid. Activity was only observed with the pThr-peptide (RRA(pT)VA), which was also used for detailed characterization for *Saci*-PP2A (Reimann *et al.* 2013). *In vivo* analysis of *Pyd. abyssi* cells grown in the presence of $^{32}P_i$ revealed six phosphorylated polypeptides between 30 and

250 kDa, which, however, could not be dephosphorylated by the *Pdab*-PP (Mai *et al.* 1998).

Archaeal PPM

The only study concerning archaeal PPMs to date was conducted by Dahche *et al.* (2009), in the euryarchaeon *T. volcanium*. The identified PPM in the genome of *T. volcanium* comprises only 218 amino acid residues; however, the comparison with the PPM from *Bacillus subtilis* revealed that all essential motifs are present. As assumed, this enzyme was Me^{2+} dependent and specific for Mn^{2+} ions, whereas Mg^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+} or Zn^{2+} had no effect or in the case of Zn^{2+}, inhibited the enzyme. Notably, *Tvo*-PPM possesses very broad substrate specificity towards pSer/pThr and also pTyr substrates, dephosphorylating all of them with a comparable rate.

Archaeal PTPs

The first archaeal PTP was characterized in 2002 from the hyperthermophilic euryarchaeon *T. kodakaraensis* KOD1 (Jeon *et al.* 2002). The biochemical characterization of the heterologously overexpressed PTP and site-directed mutagenesis revealed that the *Tko*-PTP dephosphorylates pTyr and pSer. However, no activity with pThr was observed. Mutation of the residue A_{63}, which is considered as general acid/base involved in the hydrolysis of the phosphate monoester (Zhang, Wang and Dixon 1994; Wu and Zhang 1996), resulted in an increased activity, suggesting that A_{63} is not the residue involved in the phosphate binding. The residues C_{93} and R_{99} are both part of the PTP signature motif —CMGGLGRS— (Fig. 7) and it was shown that the Cys residue is important for the formation of the phosphoryl-Cys intermediate (Denu *et al.* 1996), whereas the Arg residue is essential for the transition state and the substrate binding in general (Barford 1996). This assumption was confirmed by mutational analysis and in addition, Jeon *et al.* (2002) were able to identify three *in vivo* substrates using a trapping approach. They identified three Tyr phosphorylated proteins via immunoblotting with anti-pTyr antibodies corresponding to RtcB (part of the RNA terminal phosphatase cyclase), phenylalanine-tRNA synthetase β-chain and phosphomannomutase (PMM).

Two more PTPs were investigated both from the crenarchaeal model organisms *S. solfataricus* (Chu and Wang 2007) and *S. acidocaldarius* DSM639 (Reimann *et al.* 2013). The activity of the Sso-PTP was assayed using the general phosphatase substrate paranitrophenyl phosphate (pNPP) and the pTyr peptides A(pY)R and NK(pY)GN. The latter corresponds to the predicted phosphorylation site of the PMM, which was identified as an interaction partner/substrate for the *Tko*-PTP (Jeon *et al.* 2002). Using these substrates, *in vitro* PTP activity was confirmed. However, PP activity with pSer and pThr was not observed, revealing that the *Sso*-PTP is a conventional PTP without dual substrate specificity. Furthermore, the crystal structures of the WT *Sso*-PTP apo-enzyme and the enzyme in combination with phosphate and tungstate as well as the structures of an inactive *Sso*-PTP($C_{96}S$) mutant were solved. The overall structure resembles a compact fold of known PTPs (Cdc14B, KAPt and VHR) with a five-strand β-sheet and four α-helices, although the loop regions between $\beta2$-$\beta3$ (box 1, α-helix in Sso-PTP) and $\beta3$-$\beta4$ (box 2, helix-loop-helix *Sso*-PTP) are different (Yuvaniyama *et al.* 1996; Song *et al.* 2001; Gray *et al.* 2003) (Fig. 7). The motif Dx(25-41)HCxxGxxR(T/S) (x stands for any residue) is conserved in PTPs and dual-specific phosphatases (DSPs) and comprises the catalytic Asp and the Cys residue that act as a general acid and a nucleophile in the catalytic mechanism, respectively. Furthermore, the structure of an inactive *Sso*-PTP($C_{96}S$) in the presence of a peptide, NK(pY)YGN, revealed that the phosphate moiety of P-Tyr is bound to the P-Loop and buried in the active site surrounded by the P-loop. Additional binding of the phosphate moiety was observed to Asp_{69} (D-Loop) and Gln_{135} (Q-Loop) (Chu and Wang 2007). Gln135 is an important structural feature, since it hinders the entrance of either P-Ser or P-Thr in *Sso*-PTP. In addition, interactions between Arg_{130} and the P-loop with the bound substrate were observed. These interactions are shown by red dots in Fig. 7 and were also identified for other PTPs.

Until now, it has not been possible to distinguish between dual-specific or conventional PTPs based on the amino acid sequence alone. However, one general observation derived from available structures is that the active site of a conventional PTP is usually deeper than the active site of a dual-specific PTP for better interaction with the 'bulky' pTyr. The *in vitro* activity measurements already revealed that the *Sso*-PTP is specific for pTyr. This was also confirmed by the shape of the *Sso*-PTP active site, which allows for a better interaction with pTyr than with pSer or pThr. Potentially, the most important residue for this interaction is Gln_{135}, which hinders a proper binding of pSer or pThr substrates in the active site (Chu and Wang 2007).

In vivo evidence for phosphatase function in *Sulfolobus acidocaldarius*

Recently, the PPs from *S. acidocaldarius*, *Saci*-PP2A (see above) and *Saci*-PTP were analyzed by using a combined *in vitro* and *in vivo* approach. Deletion mutants were constructed and phenotypically characterized (i.e. growth experiments, cell size, motility assays) in comparison to the parental strain (MW001). In addition, the different *S. acidocaldarius* strains were analyzed using transcriptomics (RNAseq), phosphoproteomics and both PPs were characterized with respect to their enzymatic properties (Reimann *et al.* 2013). The characterization of the *Saci*-PPs was performed with pNPP and three phosphopeptides (NIDAIRA(pS)LNIMSR (Saci_1346), ETTYERW(pT)TITQRER (Saci_1857) and TEVGKRI(pY)RLVGDKN (Saci_1938)) identified during the phosphoproteome analysis. For the *Saci*-PP2A pSer/pThr phosphatase activity and inhibition by okadaic acid (56% activity in presence of 10 nM of okadaic acid) as well as metal dependence (highest activity with Cu^{2+} followed by Mn^{2+}, Ni^{2+}, Mg^{2+}, Cd^{2+} and Co^{2+}; complete inhibition by EDTA) was demonstrated. In contrast, the *Saci*-PTP exhibited dual substrate specificity (pSer/pThr and pTyr phosphatase) and no metal dependence or inhibition by okadaic acid was observed. The *Saci*-PTP showed nearly the same K_m value for all three tested phosphopeptides; however, the determined V_{max} value for the pTyr peptide was 30 to 131-fold higher, compared to the pSer and pThr peptide, suggesting that pTyr is the favored physiological substrate. Notably, even though *Sso*-PTP and *Saci*-PTP have 63% sequence identity they do not show the same specificity. *Saci*-PTP is a DSP, while *Sso*-PTP is solely active on pTyr residues, despite the fact that both have the essential residues important for specificity and activity (Fig. 7).

Whereas the deletion of *Saci*-PTP revealed no obvious phenotype, in the *Saci*-PP2A deletion strain, pronounced alterations in growth, cell shape and cell size were observed. The genome-wide transcriptome and phosphoproteome analysis revealed 155 genes that were differently expressed in the two mutants compared to the parental strain. A total of 801 unique phosphoproteins (1206 phosphopeptides) were identified, with a

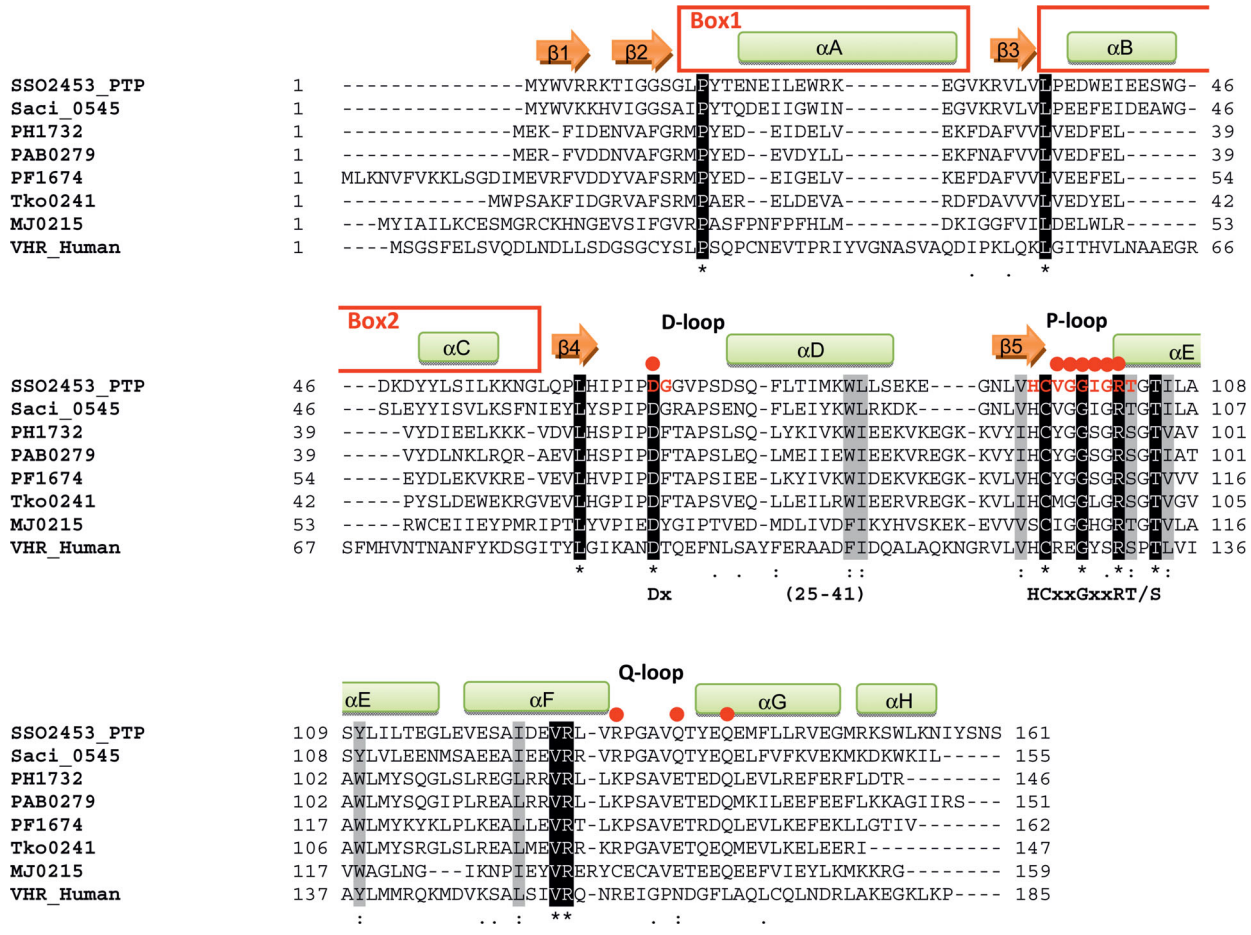

Figure 7. Amino acid sequence comparison of PTPs. Structure-based sequence alignment of the investigated PTPs from *S. solfataricus* (SSO2453), *S. acidocaldarius* (Saci_0545), *T. kodakarensis* (Tko0241) and human (VHR) as well as the uncharacterized archaeal PTPs from *P. horikoshii* (PH1732), *P. abyssi* (PAB0279), *P. furiosus* (PF1674) and *M. janaschii* (MJ0251). Secondary structure elements of the solved Sso-PTP structure are shown above the sequence with β-sheets depicted as orange arrows and α helices as green boxes (Chu and Wang 2007). The variable region of PTPs and DSPs, box 1 and box 2, are boxed red and the conserved sequence motif (Dx(25-41)HCxxGxxRT/S) is shown underneath the sequence and shown in red in the Sso-PTP sequence. The amino acid residues identified in the Sso-PTP to be involved in binding of the phosphopeptides A-pY-R and N-K-pY-G-N are marked by red dots. The alignment was generated with Clustal Omega, * conserved residues (black shading), : strong similarity between residues (gray shading),. weak similarity between residues.

significant increase in the phosphatase deletion strains. Again an unusually high number of phosphorylated Tyr residues (pSer/pThr/pTyr %-ratio of 35.6/28.1/36.2) was detected. Regulated phosphoproteins from most functional arCOGs categories were observed, including components of the motility system (archaellum), respiratory chain and regulatory proteins such as many transcriptional regulators and Ser/Thr PKs. In the *Saci*-PP2A deletion strain, the upregulation at the transcript level as well as the phosphorylation pattern resembled starvation stress response, which led to the upregulation of the archaellum. This observation was supported by the hypermotile phenotype of the mutant (see in section archaellum regulation and Fig. 3B; Reimann *et al.* 2013).

CONCLUSION

This review highlights the essential role of reversible protein phosphorylation in the regulation of basic cellular processes in Archaea and summarizes first insights into archaeal signal transduction. A striking feature in Archaea is the unusual split between the two major archaeal kingdoms, the Euryarchaeota and the Crenarchaeota, with bacterial-type TCS being only present in the Euryarchaeota, whereas protein phosphorylation on Ser, Thr and Tyr residues (via Hanks-type PKs, ePKs) is commonly distributed.

Phosphoproteome studies revealed that in common with Bacteria and Eukaryotes, for Archaea a very high number of phosphoproteins is observed (Schulz *et al.* 2014). Members of almost all arCOG categories were observed to be phosphorylated underpinning its fundamental role. An unexpected observation (so far) was the high amount of Tyr phosphorylation specifically in *Sulfolobus* species and seemingly absent in *Halobacterium salinarium*. In Archaea, no bona fide Hanks-type Tyr PKs or BY-kinases were identified so far, raising questions about the kinases involved in Tyr phosphorylation. This is further underpinned by the high number of phosphoproteins and relatively low amount of ePKs and PPs in Archaea, suggesting the presence of other so far unknown enzymes in Archaea.

In general, Tyr phosphorylation is regarded as an invention of Eukaryotes to regulate more complex cellular networks. In the light of the newly proposed two-domain tree of life with the origin of Eukaryotes in the archaeal TACK superphylum, this might represent a shared phylogenetic trait. Therefore, it is of special importance to identify and study more pathways in Archaea in which regulation by reversible protein phosphorylation plays a role and to understand their mechanisms.

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