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ORIGINAL PAPER

Archaeal transcriptional regulation of the prokaryotic KdpFABC complex mediating K^+ uptake in *H. salinarum*

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Abstract The genome of the halophilic archaeon *Halobacterium salinarum* encodes the high-affinity ATP-dependent K^+ uptake system Kdp. Previous studies have shown that the genes coding for the KdpFABC complex are arranged in a *kdpFABCQ* gene cluster together with an additional gene *kdpQ*. In bacteria, expression of the *kdpFABC* genes is generally regulated by the dedicated sensor kinase/response regulator pair KdpD/KdpE, which are absent in *H. salinarum*. Surprisingly, *H. salinarum* expresses the *kdp* genes in a manner which is strikingly similar to *Escherichia coli*. In this study, we show that the halobacterial *kdpFABCQ* genes constitute an operon and that *kdpFABCQ* expression is subject to a complex regulatory mechanism involving a negative transcriptional regulator and is further modulated via a so far unknown mechanism. We describe how the regulation of *kdp* gene expression is facilitated in *H. salinarum* in contrast to its bacterial counterparts. Whereas the Kdp system fulfils the same core function as an ATP-driven K^+ uptake system in both archaea and bacteria, the different mechanisms involved in the regulation of gene expression appear to have evolved separately, possibly reflecting a different physiological role of ATP-driven K^+ uptake in halophilic archaea.

Keywords Haloarchaea · *Halobacterium* · Potassium uptake · KdpFABC · KdpQ · KdpD · Gene regulation

Abbreviations

TFB	Transcription factor B
BRE	Transcription factor B recognition element
TBP	TATA-box binding protein
INR	Transcription initiation region
Usp	Universal stress protein
RT	Reverse transcriptase

Introduction

Organisms living in high-salt environments encounter an extraordinary challenge in maintaining adequate cellular water levels. Halophilic archaea balance their cytoplasm by the accumulation of KCl at concentrations at least as high as the NaCl concentration in their environment (Oren 1999). The genome of *Halobacterium salinarum* encodes a variety of different K^+ uptake systems including low-affinity K^+ channels (PchAB) and K^+/H^+-symporters (TrkAH), which are driven by the membrane potential, as well as the high-affinity ATP-driven K^+ uptake system Kdp (Pfeiffer et al. 2008). Although the regulation of K^+ uptake is essential to maintain the balance between the cellular K^+ concentration and external osmolality, very little is known about the underlying transcriptional regulation of the corresponding uptake systems in halophilic archaea.

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In the mesophilic bacterium *Escherichia coli,* the low-affinity K^+ uptake systems Kup and Trk are constitutively expressed, whereas the high-affinity Kdp system is regulated at the level of transcription (Epstein 2003). A similar coordination of K^+ uptake systems can be found in *H. salinarum*. Transcription of the genes coding for the K^+ uptake systems Trk and Pch has been shown to remain rather unaffected by changes in medium salinity (Coker et al. 2007).

In previous studies, the expression of the *kdp* genes was found to be highly induced in *H. salinarum* upon K^+ depletion. The structural genes (*kdpFABC*) coding for the KdpFABC transport ATPase appear to be co-expressed with an additional gene *kdpQ*, thereby forming a *kdpFABCQ* gene cluster (Fig. 1a) (Strahl and Greie 2008). High K^+ concentrations in the medium were found to repress transcription, whereas a moderate level of expression occurs in the presence of extracellular K^+ concentrations below 20 mM. Below 250 µM, *kdp* gene expression is highly induced (up to 860-fold at 20 µM external K^+). When the cells cease to grow due to K^+ limitation, the expression level was found to decrease in the stationary growth phase independent of the medium K^+ concentration (Strahl and Greie 2008). This rather complex expression pattern already indicated that expression of the *kdpFABCQ* genes is under control of an intricate transcriptional regulation.

In *E. coli*, *kdp* gene expression also occurs under conditions of low external potassium concentrations (Hamann et al. 2008; Ballal et al. 2007). Hence, the Kdp complex apparently shares the same core function in K^+ uptake upon low medium K^+ concentrations in both bacteria and archaea. In contrast, the expression of the *E. coli kdpFABC* operon is mediated by the gene products of the adjacent *kdpDE* operon, which constitute the dedicated two-component sensor kinase/response regulator system KdpD/KdpE (Fig. 1b) (Rhoads et al. 1978; Gaßel et al. 1999; Polarek et al. 1992; Jung and Altendorf 2002). Although homologues of *kdpDE* are absent in the genome of *H. salinarum*, the *kdp* gene expression patterns in *H. salinarum* and *E. coli* upon K^+ limitation are virtually identical (Strahl and Greie 2008). The only similarity with respect to possible regulatory effectors can be found in a region within KdpD that comprises structural homologies to universal stress proteins (Usp) (Siegele 2005; Zimmann et al. 2007). Sequence analysis and structural homology modelling identified the halobacterial KdpQ as a tandem Usp protein, which stimulates *kdpFABCQ* gene expression under inducing conditions (Strahl and Greie 2008).

This work aims to elucidate how the *kdpFABCQ* gene expression is facilitated in *H. salinarum* and how it compares to physiology and regulatory mechanisms found in its bacterial counterparts.

Materials and methods

Strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Tables S1 and S2. Primers are listed in Table S3.

H. salinarum R1 was grown in rich media as described for *Halobacterium* sp. NRC-1 (American Type Culture Collection medium 2185). To grow *H. salinarum* strains in medium with different initial K^+ concentrations, cultivation was carried out as described by Strahl and Greie (2008). *Halobacterium* cultures were grown aerobically under illumination at 37°C. If required, the media were supplemented with 20 µg/ml of Simvastatin, which was

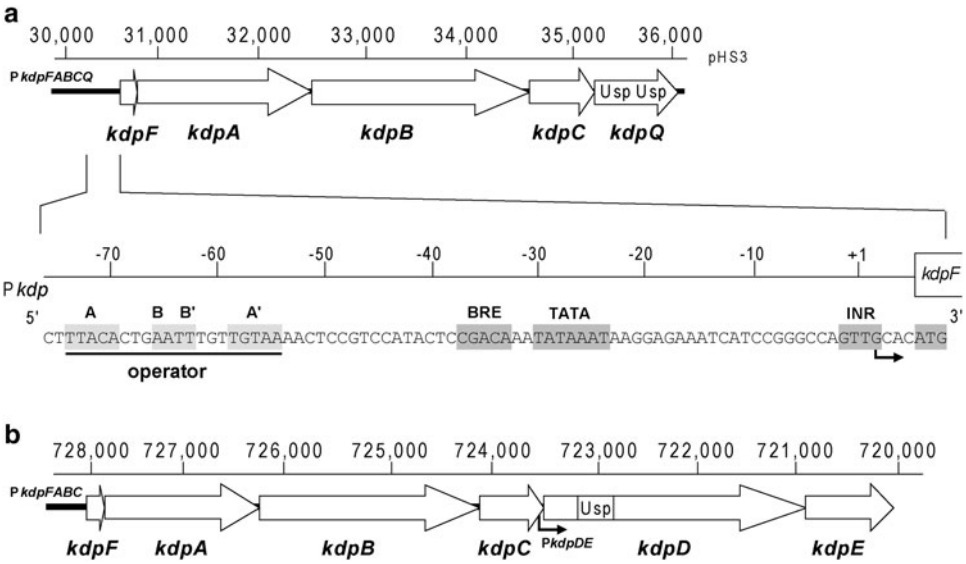

Fig. 1 **a** Organization of the *kdpFABCQ* operon and its promoter (P*kdp*) in *Halobacterium salinarum*. The basal transcription elements BRE, TATA and INR are indicated. The palindromic sequence of the operator is denoted by *A/A′* and *B/B′*. **b** Organization of the *kdpFABC* operon and the adjacent *kdpDE* operon in *Escherichia coli*. The *numbers* indicate the localization of the genes on the extrachromosomal pHS3 of *H. salinarum* R1 (Pfeiffer et al. 2008) and on the *E. coli* chromosome (Blattner et al. 1997)

ethanol-extracted from solid tablets (Ratiopharm). If not stated otherwise, samples for subsequent experiments were taken from *H. salinarum* cultures grown under K^+-limiting conditions (3 mM initial K^+) at an optical density of 0.9–1, where cells enter the stationary growth phase due to K^+ limitation (Strahl and Greie 2008). To compare gene expression profiles of cells grown under K^+-limiting conditions with that of cells grown under non-limiting conditions (100 mM initial K^+), the samples were taken at the same point of time.

Transformation of *H. salinarum* was carried out by use of polyethylene glycol 600 as described by Cline et al. (1989). For *bgaH*-encoded *β*-galactosidase activity studies, corresponding plasmids were transformed into *H. salinarum* R1 *ΔkdpFABC*. Plasmids for *kdpFABCQ* expression studies were transformed into *H. salinarum* R1 *ΔkdpFABCQ*.

Construction of mutants

In order to generate a P*kdp*::*bgaH* fusion construct, a 210-bp region located immediately upstream of the *kdpF* start codon was fused to the *bgaH* gene via two-step PCR technique on pHJS1 as template (Fig. S1). The 210-bp P*kdp* region was amplified by use of Phusion® High-Fidelity DNA Polymerase (Finnzymes) via primers 1 + 2, and a 490-bp *bgaH* fragment was amplified via primers 3 + 4. The resulting P*kdp*::*bgaH* fragment was restricted via an introduced *Pst*I restriction site and the restriction site *Eco*RI present in *bgaH* and subsequently ligated into the accordingly digested pMKK100 multiple cloning site (MCS) upstream of *bgaH*, resulting in plasmid pDSK1. In this construct, the *bgaH* fragment is directly fused to the *kdpF* start codon. Since pMKK100 carries no origin of replication for *H. salinarum*, two additional 450 bp sequences naturally framing the *kdpFABCQ*-operon were previously introduced in pMKK100 for recombination. The adjacent 450 bp sequences were restricted in pHJS4 via *Nco*I and *Hind*III and accordingly cloned into the MCS of pMKK100. Further promoter truncations in pDSK1 were generated by PCR using primers 5–22 together with primer 4, again cloned via *Pst*I and *Eco*RI, resulting in plasmids pDSK2–19.

The deletion of the operator was basically constructed like the pDSK plasmids, briefly by two-step PCR on pDSK1 using primers 1 and 23 together with primers 24 and 4, resulting in plasmid pHJS9.

RNA extraction, cDNA synthesis, RT-PCR, and real-time RT-PCR

Total RNA of *H. salinarum* R1 strains was isolated via the RNeasy mini kit (Qiagen) following the manufacturer's instructions, except for the use of lysozyme in TE buffer. To remove residual DNA contaminations, DNase I (NEB) digestion was performed on 140 ng diluted RNA. Subsequently, 70 ng total RNA was reversely transcribed via random hexamer primers by use of the First Strand cDNA Synthesis Kit (Fermentas). In order to verify that all *kdp* genes are transcribed as a polycistronic mRNA, six primer sets for a RT-PCR approach were designed to amplify overlapping products. Primer sets *kdpF*/*kdpA* (25 and 26), *kdpA*/*kdpB* (27 and 28), *kdpB*/*kdpB* (29 and 30), *kdpB*1/*kdpB*1 (31 and 32) as well as *kdpB*/*kdpQ* (33 and 34), generate 1000 bp products, whereas primer set *kdpQ*/*kdpQ* (35 and 36) generates a 750 bp product. DNaseI-treated RNA (without reverse transcription) was used to check for genomic DNA contamination. PCR amplification was carried out with Phusion® High-Fidelity DNA Polymerase under standard reaction conditions.

Real-time RT-PCR analyses were performed in triplicate by use of primers 37 and 38 binding in *kdpC* and *kdpQ*, respectively. Primers 39 and 40 targeted at the *H. salinarum* housekeeping gene *rpoA1* were used to normalize the calculated C_t values. Basically, real-time RT-PCR was carried out as described by Strahl and Greie (2008).

Northern blot analysis

In order to generate a digoxigenin (DIG)-labelled RNA probe, a PCR product containing the T7 promoter followed by 350 bp of *kdpA* (*kdpA*: 669–1022 bp) as hybridization target was generated from pHJS1 via primers 41 and 42. RNA was transcribed in vitro from the PCR template by use of 100 U of T7 RNA polymerase (NEB) in a reaction mixture containing transcription buffer (NEB) and DIG RNA labelling mix (Roche). The reaction was incubated for 2 h at 37°C, followed by RNA precipitation with LiCl and ethanol. Fifty micrograms of total RNA from *H. salinarum* R1 and *H. salinarum* R1 *ΔkdpFABCQ* grown under K^+-limiting conditions (3 mM initial K^+) together with 50 ng of DIG-labelled RNA molecular weight marker (Roche) were separated under denaturing conditions on a 1.2% (w/v) agarose/8.5% (v/v) formaldehyde–MOPS gel and transferred to a positively charged nylon membrane (Roche) by a capillary blot setup using 20× SSC buffer (3 M NaCl, 0.3 M sodium citrate). After incubation at 120°C for 30 min, the membrane was preincubated and hybridized under stringent conditions at 68°C in 50% (v/v) formamide, 5× SSC, 0.1% (w/v) *N*-lauroylsarcosine, 0.02% SDS (w/v), 2% (w/v) blocking solution (Roche) with the DIG-labelled *kdpA* RNA probe. Washing steps and application of alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche), as well as use of the chemiluminescence substrate CDP-*Star* (Roche), was performed according to manufacturers' instructions. Chemiluminescent signals were detected on Kodak Biomax XAR films.

Mapping of the transcription start site via primer extension assay

Primer extension was performed with 100 µg of total RNA from *H. salinarum* R1 by use of 100 fmol of the 5′-end 6′-FAM fluorescent-labelled primer 43 or the 5′-end ROX-labelled primer 44 by use of the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). The binding site of the primers was located 294 bp downstream of the *kdpF* start codon. The resulting cDNA product was separated from the fluorescent primer by use of the DyeEx Kit (Qiagen). Electrophoresis was performed on a 4.8% (w/v) polyacrylamide–urea gel PAGE-Plus 40% conc. (Amresco) using an ABI PRISM® 377 Genetic Analyzer (Applied Biosystems, Darmstadt), which was pre-run at 1,000 V until the gel temperature reached 51°C. Each cDNA sample was dissolved in a solution of 3 µl formamide (Amresco, Ohio), 0.5 µl GeneScan®-500 Rox™ size standard (Applied Biosystems) and 2 µl of loading buffer (Applied Biosystems). Samples were heated to 95°C for 3 min prior to application. Electrophoresis was carried out for 2 h at a constant voltage of 3.0 kV. DNA fragment sizes were determined by use of the GeneScan® Analysis Software version 3.1 (Applied Biosystems) using Filter Set A.

DNA microarray analysis

H. salinarum was grown under K^+ limiting and non-limiting conditions as described earlier using media containing 3 and 100 mM initial K^+, respectively. Extraction and DNaseI digestion of total RNA was performed as described earlier. Total RNA was reversely transcribed into Cy5- or Cy3-labelled cDNA by use of the CyScribe First-Strand cDNA Labeling Kit (GE, Amersham Biosciences) with the enclosed random nonamer primers and Cy5-/Cy3-dUTP. RNA was alkaline hydrolyzed, and the cDNA was purified and concentrated as described previously (Zaigler et al. 2003). The analysis of corresponding RNA 'pairs' (growth in 3 and 100 mM K^+) was designed as a dye-swap experiment, in which the cDNA fluorescence labels were swapped in the second run of cDNA synthesis. The two pairs of fluorescent cDNA were hybridized to DNA microarrays according to Zaigler et al. (2003). The microarrays were produced as described in detail by Twellmeyer et al. (2007). Hybridized DNA microarrays were evaluated by use of a GenePix 4000B microarray scanner (Biozym Scientific) according to Wende et al. (2009). All microarray data have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/, accession number E-MEXP-3225).

β-Galactosidase activity assays

β-Galactosidase activities in cell lysates were measured in triplicate using the ONPG assay as described by Holmes et al. (1997). Measurements were carried out in 96-well microtiter plates (Falcon) in an ELISA-Reader SLT 340 (ATTC). After preliminary controls for the dependency between optical density and protein concentration, the culture optical density measurements were taken to calibrate the amount of cell material used in the assay.

Phylogenetic computation

Multiple sequence alignments and pair-wise distance matrices of 89 bacterial and eight archaeal homologues of KdpB were calculated using ClustalW (Thompson et al. 1994). An unrooted radial phylogenetic tree was generated with the Phylodraw software (Choi et al. 2000) using the ClustalW distance matrix and the neighbour joining algorithm (Saitou and Nei 1987).

Determination of medium K^+ concentrations

K^+concentrations in the growth medium were measured by flame emission photometry using an Eppendorf ELEX 6361 flame photometer in a buffer containing 5 mM of CsCl and 1.5% (w/v) trichloroacetic acid.

Results

Determination of the *kdpFABCQ* transcription start site

Based on sequence analysis, the promoter region (P*kdp*) of the *kdpFABCQ* gene cluster resembles a typical archaeal promoter (Fig. 1a). A well-conserved TATA box element centred at 32 bp 5′ of the *kdpF* start codon, as well as a transcription factor B binding site (BRE) located 2 bp upstream of the TATA box, could be predicted (Strahl and Greie 2008; Palmer and Daniels 1995; Brenneis et al. 2007). Furthermore, a putative transcription initiation region (INR) 5′-GTTG-3′ marking the transcription start site is located 4 bp upstream of the *kdpF* start codon. Although the INR motif does not correspond to the common consensus sequence 5′-TCG/A-3′, it is identical to the well-defined INR element of the bacterio-opsin promoter (Gropp et al. 1995).

Archaeal genes organized into operons are commonly expressed as a leaderless transcript (Tolstrup et al. 2000; Slupska et al. 2001). In order to verify that this also applies for a putative *kdpFABCQ* operon and to identify the start site of transcription, primer extension assays on total RNA with fluorescently labelled primers were carried out. The primer binding site was chosen 294 bp downstream of the *kdpF* start codon. The use of a 6′-FAM-labelled primer resulted in a 298 bp run-off product as indicated by a distinct blue band on the sequencing gel together with a pronounced blue peak in the electropherogram (Fig. 2a, b).

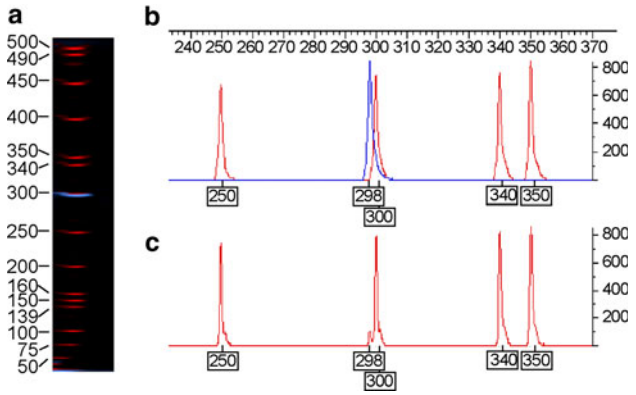

Fig. 2 Primer extension assay for the determination of the transcription start site of the *kdpFABCQ* transcript. **a** Image of the sequencing gel. The *red bands* indicate the GeneScan®-500 Rox™ internal lane standard, whereas the *blue band* represents the FAM-labelled *kdp* run-off product. **b**, **c** Electropherograms of the primer extension samples using a 5′-end 6′-FAM and a 5′-end ROX fluorescent-labelled primer, respectively. The binding site of both primers was chosen 294 bp downstream of the *kdpF* start codon. *Red peaks* indicate the GeneScan®-500 Rox™ internal lane standard (fragment length of each peak is given in base pairs). The signal at 298 bp in both assays represents the *kdp* run-off product (Color figure online)

To exclude a size effect of the 6′-FAM label with respect to the ROX size standard, the primer extension assay was verified by use of a ROX-labelled primer. Here, the run-off product confirmed the 298 bp fragment length (Fig. 2c). These results demonstrate that the transcription of the *kdpFABCQ* genes is initiated 4 bp upstream of the *kdpF* start codon, most likely utilizing an INR homologous to that of the bacterio-opsin promoter.

The *kdpFABCQ* genes are transcribed as a single polycistronic mRNA

The *kdpFABCQ* genes are apparently organized in an operon corresponding to a polycistronic transcript length of 5469 bp. The single genes are separated by intergenic regions of not more than two base pairs. Furthermore, a conserved promoter sequence can only be found upstream of the first gene *kdpF*. Experimental verification of the *kdpFABCQ* operon was done via RT-PCR by use of different primers amplifying overlapping products (Fig. 3a). Clearly defined bands of proper size were obtained for every primer set with cDNA as template, which are absent in the negative control (DNaseI-treated RNA without reverse transcription) (Fig. 3b). These findings are further supported by a Northern blot analysis using a probe binding in *kdpA* (Fig. 3c), in which a band at ~5.5 kb can be identified, corresponding to the full-length *kdpFABCQ* transcript. This band cannot be detected in the *kdp* deletion strain (*H. salinarum ΔkdpFABCQ*) even in the case of fourfold extended exposure time (Fig. 3d). A putative terminator or a potential mRNA processing site can be found 85–110 bp downstream of *kdpQ* (Fig. S2), which is well in accordance with the determined length of the *kdpFABCQ* transcript. Hence, the *kdpFABCQ* genes are transcribed as a polycistronic unit and, thus, form an operon.

Analysis of putative *trans*- and *cis*-acting regulatory elements

Transcriptional regulators are frequently encoded in the near vicinity of the genes they regulate, especially in case of bacterial *kdp* operons (Ballal et al. 2007). As discussed by Strahl and Greie (2008), a further regulatory component in addition to KdpQ is involved in the transcriptional regulation of the *kdp* operon in *H. salinarum*. However, sequence analysis revealed that no putative transcriptional regulators are encoded in a region 10 kbp up- or downstream of the *kdpFABCQ* operon. Since in *E. coli* and other bacteria, *kdpFABC* expression is mediated by a dedicated two-component sensor kinase/response regulator system KdpDE, we analyzed the two-component systems of *H. salinarum* for their potential to regulate the halobacterial *kdp* genes. Beside the well-known Che two-component sensory systems, the genome of *H. salinarum* R1 encodes thirteen sensor kinases but only three response regulators of the DNA-binding type (Pfeiffer et al. 2008; Wende et al. 2009). The KdpD sensor kinase, which is essential for the regulation of the bacterial *kdp* operons, contains a conserved and unique N-terminal domain (KdpDN), which allows a clear separation between KdpD and other sensor kinases (Heermann et al. 2003). Proteins comprising this distinctive domain are absent in *H. salinarum*, suggesting the absence of a KdpD homologue. In addition to the sensor kinase, all bacterial Kdp systems rely on the presence of a corresponding response regulator. Therefore, we tested deletion strains of all three predicted DNA-binding response regulators present in *H. salinarum*: *hlx1* (OE3854), *hrg* (OE2334), and *hlx2* (OE2086) (Wende et al. 2009), for their effect on *kdpFABCQ* expression. However, these deletion strains featured *kdp* gene induction patterns comparable with the *H. salinarum* wild type (Fig. S3). Hence, the halobacterial *kdp* genes are not regulated via the two-component systems involving these response regulators.

A truncation analysis of the promoter was carried out in order to experimentally analyze the predicted conserved promoter elements of P*kdp* and to gain insight how the *kdp* genes are regulated in *H. salinarum*. A 206 bp region upstream of the *kdpFABCQ* transcription start site was initially selected as promoter sequence most likely containing all *cis*-acting elements and not overlapping with the neighbouring gene (*trkA2*) located upstream of the *kdp*

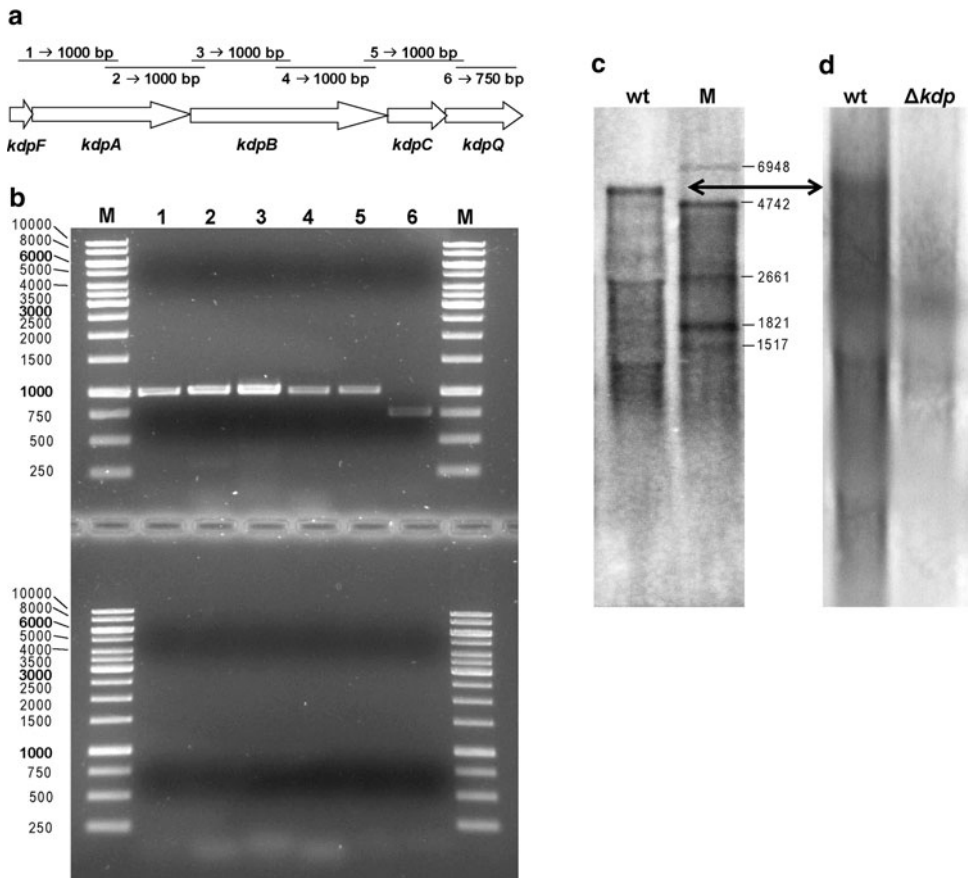

Fig. 3 Analysis of the polycistronic *kdpFABCQ* transcript. **a** Location of the primers amplifying overlapping fragments of the *kdpFABCQ* operon in RT-PCR. **b** Analysis of RT-PCR amplification products; *upper panel* cDNA as template (RT-PCR), *lower panel* RNA after DNaseI digestion (no RT) as negative control to exclude DNA contamination in RNA preparations. *M* GeneRuler™ molecular weight marker, 1 kb DNA ladder (Fermentas). **c** Northern blot analysis of the putative *kdpFABCQ* operon. *M* DIG-labelled RNA molecular weight marker II, *wt* RNA of *H. salinarum* R1. **d** Fourfold extended exposure time of *H. salinarum* R1 RNA (wt) and RNA of *H. salinarum* R1 *ΔkdpFABCQ* (Δ*kdp*). The 5.5-kb polycistronic *kdpFABCQ* transcript is highlighted by an *arrow*

operon. To identify the promoter regions required for the expression and regulation of the *kdpFABCQ* operon, translational fusions between the halophilic *β*-galactosidase gene *bgaH* (Patenge et al. 2000) and truncated *kdpFABCQ* promoters were constructed and inserted into the genome by use of an integrative vector background. To minimize possible polar effects on neighbouring genes, the integrative vectors were designed in a way that all non-coding sequences surrounding the *kdp* operon remained unaffected. The different promoter fusions were examined under both inducing and non-inducing conditions, i.e., low (3 mM) and high (100 mM) medium K^+, respectively, and corresponding *bgaH* expression levels were measured quantitatively as *β*-galactosidase activities (Fig. 4). Since fluctuations in overall activity are frequently observed in *β*-galactosidase activity measurements from experiment to experiment (Fig. 4a), the ratio of non-induced to induced expression was taken for direct comparison of induction levels, whereby the induced expression rates were set to 100% (Fig. 4b). Extensive truncations coding for −25 to −36 bp promoter fragments exhibited only barely detectable levels of *β*-galactosidase activities comparable with that of the negative control (*H. salinarum* without *bgaH*). Hence, in these constructs the probable basal transcription elements are severely affected. These findings are well in accordance with the predicted location of the TATA-box (centred at −28 bp) and the consensus BRE (−34 to −38 bp) (Fig. 1). Less extensively truncated promoters comprised clear differences regarding their ability to respond to non-inducing growth conditions. −206 and −96 bp promoter fragments exhibited a strong expression under inducing conditions and only low levels under non-inducing conditions, thereby corresponding to wild type. In contrast, further truncation of the promoter ranging from −71 to −39 bp resulted in significantly increased expression levels even under non-inducing conditions. Hence, a negative regulatory element appears to be affected when the promoter sequence is shorter than −71 bp. Sequence analysis of this region revealed a palindrome located from −74 to −55 bp (Fig. 1a), which represents a typical binding site of a helix-turn-helix-type transcriptional regulator. In promoter truncations −71 or less, this palindromic sequence is significantly disrupted or absent, thereby resulting in a loss of its putative function. To confirm this potential operator sequence, a site-directed deletion of this element was carried out (−206Δop). Corresponding constructs were analyzed by both real-time RT-PCR and *β*-galactosidase fusion studies (Fig. 5). Whereas under inducing conditions, the −206Δop construct again exhibited expression levels comparable to the full-length

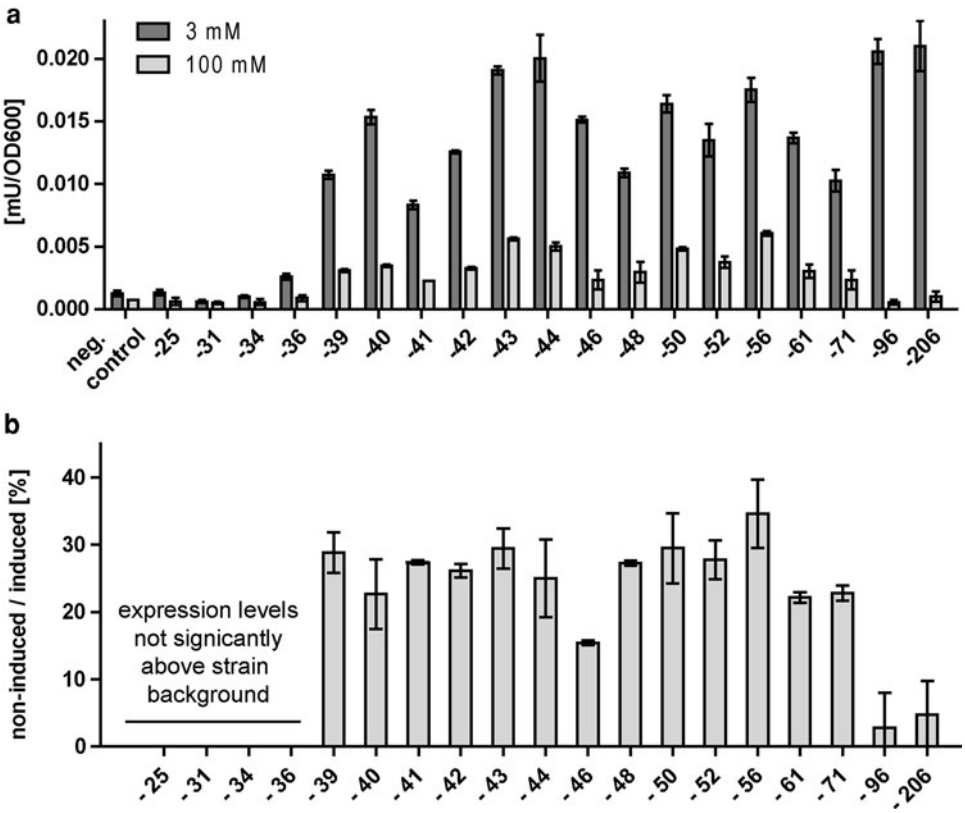

Fig. 4 **a** *β*-Galactosidase activities of *H. salinarum* R1 *ΔkdpFABC* encoding different P*kdp*::*bgaH* fusions. Samples were taken from cultures grown under inducing (3 mM K^+) and non-inducing (100 mM K^+) conditions. *Numbers* indicate truncations of the promoter with respect to the transcription start site. **b** Ratio of non-induced to induced *bgaH* expression levels (non-induced expression set to 100% representing constitutive expression)

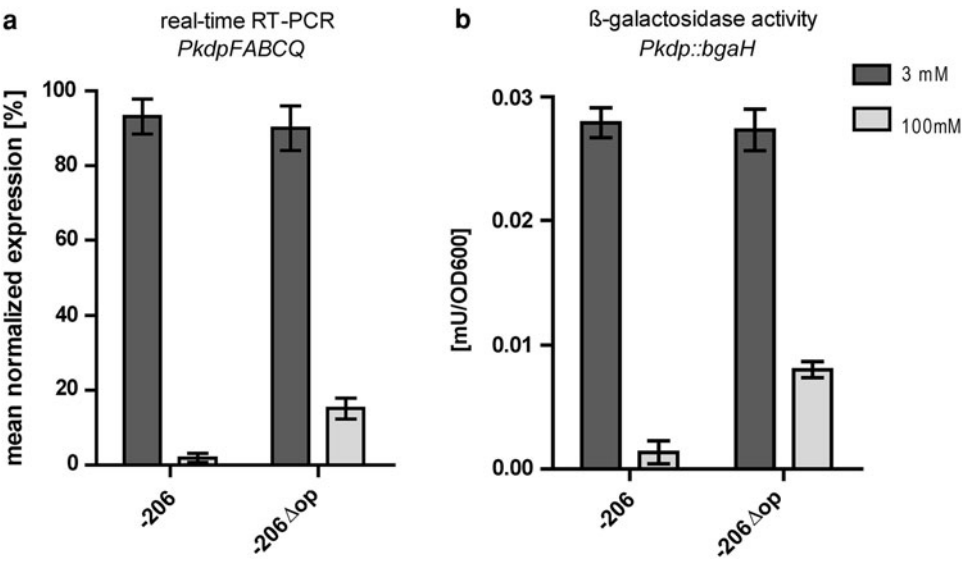

Fig. 5 **a** Normalized expression levels of plasmid-encoded *kdpFABCQ* expressed in *H. salinarum* R1 *ΔkdpFABCQ* from the full-length promoter (−206) and from the promoter comprising a deletion of the operator (−206Δop) measured by real-time RT-PCR. **b** *β*-galactosidase activities of corresponding transcriptional fusions to *bgaH* in *H. salinarum* R1 *ΔkdpFABC*

promoter (−206), expression was again found to be strongly increased under non-inducing conditions, in which the −206 promoter is efficiently repressed. Hence, this palindromic element can be interpreted as an operator which is involved in a negative regulation of *kdpFABCQ* operon expression. Surprisingly, the expression levels of the −206Δop construct under non-inducing conditions were only ∼20% of the induced level but not a constitutive 100%, as one would expect if regulation solely occurs via this operator. Thus, an additional mode of regulation seems to be present. A further regulatory element located in the coding region of *kdpFABCQ* can be excluded since the expression levels of the translational *bgaH*-fusion to the *kdpF* start codon were comparable with that determined by real-time RT-PCR in the native context. Therefore, the region between the transcription start site and the putative TATA-box/BRE was further analyzed by use of a scanning mutagenesis approach for a potential regulatory element.

However, no obvious changes in the non-induced expression levels could be derived from these data, which would be indicative of the presence of an additional operator site (data not shown).

Global transcriptional response of *H. salinarum* R1 to K^+ limitation

Since potassium is essentially involved in a variety of cellular processes, external K^+ limitation is likely to have broad cellular effects. Under these conditions, the cells have to adapt to lower intracellular K^+ concentrations, and cells cease to grow when K^+ uptake is not sufficient (Strahl and Greie 2008). Hence, alterations in transcription patterns not only relating to K^+ uptake systems are most likely to occur. Therefore, *H. salinarum* cells grown under K^+-limiting and non-limiting conditions were subjected to DNA microarray analysis (Table S4). A total number of 35 genes were found to be down-regulated and 26 genes up-regulated in response to K^+ limitation. As expected, *kdpA*, *kdpB*, *kdpC*, and *kdpQ* are highly up-regulated. In addition to the *kdp* genes, the histidine kinase *ark*, the genes *trkA2* and *trkA5*, as well as a remarkably high number of unknown genes, are also induced under these conditions. TrkA homologues bind to and regulate the activity of the low-affinity TrkH K^+-uptake system (Schlösser et al. 1993). Hence, the Trk-systems seem to undergo at least some adaptation to low K^+ conditions via regulation on the protein level. In contrast, a high number of transposons together with genes coding for the A_1A_O-ATPase are down-regulated. The latter finding suggests that K^+ limitation also has a significant impact on the bioenergetic status of the cell.

Discussion

The transcriptional regulation of P*kdp* comprises interplay of three different factors. The majority of the regulatory input is facilitated by a negative regulation based on an operator located upstream of the putative conserved promoter elements. An additional and so far unknown factor is responsible for a minor regulation, which appears to be independent from this operator. At last, the expression level from P*kdp* is further regulated by the last gene of the operon, *kdpQ* (Strahl and Greie 2008)

With respect to the primary operator-dependent regulation, the location of the operator three DNA helical turns upstream of the putative conserved promoter elements appears to be rather distant in order to exhibit a direct inhibitory function on transcription initiation. However, a corresponding location of a regulatory element has already been described for the archaeon *Methanocaldococcus jannashii* (Ouhammouch et al. 2005). In this organism, the transcriptional activator Ptr2 was shown to bind at two activator sites located 21 and 31 bp upstream of the putative TATA-box and is, nevertheless, still able to interact with the TATA-box binding protein (TBP). Hence, a direct influence of a regulatory protein binding three helical turns upstream of the conserved promoter elements is feasible in archaeal promoters. Furthermore, a rather distant binding site can be straddled by oligomerization of repressor molecules as described for the transcriptional regulators ORF80 of the archaeon *Sulfolobus islandicus* (Lipps et al. 2001) and Ss-LrpB of *Sulfolobus solfataricus* (Peeters et al. 2004). In case of the *kdp* operon of *H. salinarum*, further elucidation of the underlying mechanism requires the identification and purification of the repressor. Despite several attempts involving genetic screens and DNA affinity chromatography, we have so far not been able to identify a regulatory protein that binds to the *kdp* operator.

Although the primary but operator-independent regulatory component has a smaller impact than the *kdp* operator, it is still responsible for an approximately sixfold induction under inducing conditions. Within the *kdp* coding sequence and the entire *kdp* promoter region (except the TATA box and BRE), no further *cis*-active regulatory elements could be found. However, a *cis*-active element directly overlapping with the TATA box and BRE can not be ruled out. Another potential regulatory mechanism could be accomplished by modulation and/or differential use of TBPs and TFBs under K^+-limiting conditions. Halophilic archaea encode several homologues of the basal transcription factors (Pfeiffer et al. 2008; Hartman et al. 2010) and the role in modulation of gene expression in a process analogous to bacterial sigma factors has been postulated (Facciotti et al. 2007; Coker and DasSarma 2007; Teufel et al. 2008). However, further studies are required to verify this hypothesis.

Alongside the primary transcriptional regulation, KdpQ acts as a secondary transcriptional activator, which is co-transcribed with the structural *kdp* genes (Strahl and Greie 2008). Since its own expression is completely repressed under non-inducing conditions, it can only display its function as a positive co-regulator once other regulatory mechanisms allow its expression from the tightly controlled *kdp* promoter.

In addition to the specific induction of the *kdpFABCQ* operon, DNA microarray analysis revealed that potassium depletion does not only affect systems directly involved in K^+ homeostasis. Among the high number of genes up-regulated upon K^+ limitation is the *ark* histidine kinase. Although deletion of the corresponding dedicated response regulator *hrg* had no effect on *kdpFABCQ* expression, it is still tempting to speculate that this sensor kinase could somehow be involved in *kdp* expression. Furthermore, a significant number of genes with unknown function are regulated in response to K^+ limitation and could, thus,

potentially be involved in the transcriptional regulation of the *kdpFABCQ* operon. However, it is unlikely that one of the corresponding gene products is directly binding to the identified operator since sequence analysis of these genes revealed no resemblance to any known classes of DNA-binding transcriptional regulators.

A number of 44% of the genes up-regulated upon K^+ limitation are clustered on the extrachromosomal replicon pHS3 (Fig. S4). Since archaea are able to compress their DNA into chromatin (Sandman and Reeve 2005), changes in chromatin condensation could exert a regulatory effect on a potential K^+-responsive gene cluster. However, P*kdp*::*bgaH* reporter gene fusions integrated at various regions within the genome of *H. salinarum* resulted in comparable expression levels in either case (Fig. S5), thus arguing against such a notion. However, a sequence homologous to the P*kdp* operator can also be found within the promoter of a neighbouring operon (*OE5065R* to *OE5067R*), which is also up-regulated upon K^+ limitation. This putative operator is located 21 to 41 bp upstream of the first gene *OE5067R* and, thus, at a position within the predicted promoter. It is tempting to speculate that both operons could be linked by a shared regulatory protein, thereby forming a K^+-responsive regulon.

Although the patterns of gene expression upon K^+ limitation are similar in *H. salinarum* and *E. coli*, the underlying regulatory mechanisms are completely different. Homologues of the two-component system KdpD/KdpE are absent in *H. salinarum* and the gene induction in *Halobacterium* is based on negative regulation in contrast to the bacterial counterparts. A phylogenetic computational analysis of bacterial and archaeal Kdp complexes revealed an individual archaeal clade clearly separated from their bacterial counterparts. The *kdp* genes could either be already present as the phylogeny of bacteria and archaea branched, or they could be transferred via lateral gene transfer on a very early stage. Exceptions to this are two species of the order Methanomicrobiales. These appear to have obtained the Kdp complex from bacteria independently from other archaea, since their Kdp subunits are more homologous to bacterial Kdp systems. Interestingly, these two species are the only archaea so far known to encode homologues of the bacterial sensor kinase KdpD.

A likely explanation for different mechanisms in the regulation of *kdp* gene expression is that both regulatory systems have co-evolved in bacteria and archaea in order to fulfil the same core function, i.e., ATP-dependent K^+ uptake; however, in a different physiological context. Although the Kdp system responds to K^+ limitation in both domains of life, its physiological relevance could be quite different in *H. salinarum* and *E. coli*. Bacteria occasionally encounter extreme K^+-limiting environments, in which high-affinity K^+ uptake systems are essential to promote growth. Hence, in *E. coli*, the Kdp complex facilitates K^+ uptake under conditions where the low-affinity uptake systems are not sufficient to promote growth (Ballal et al. 2007). In contrast, *H. salinarum* thrives in hypersaline environments with K^+ concentrations well above 100 mM (Oren 1994) and, thus, there is no need for a high-affinity K^+ uptake system. Therefore, it appears feasible that *H. salinarum* requires the Kdp complex not for reasons of affinity, but rather due to its ATP dependency. In order to keep up with the high intracellular K^+ requirements of at least 2.8 M (Strahl and Greie 2008), ATP-driven K^+ uptake would be essential under conditions where K^+ uptake cannot solely be driven by the membrane potential. The extraordinary high level of cytoplasmic K^+ results in a potassium potential of more than +300 mV in the presence of low medium K^+ (Strahl and Greie 2008). *H. salinarum* is able to generate and maintain a membrane potential of up to −270 mV upon illumination at its best (Michel and Oesterhelt 1980). Any drop in membrane potential would readily increase the opposing potassium potential over the membrane potential, thus preventing potential-driven K^+ uptake. Since it is, thus, not external K^+ limitation, which increases the potassium potential over the membrane potential, *kdp* gene expression is feasible in *H. salinarum* under conditions of low membrane potential even at rather high constant external K^+ concentrations. Hence, the different regulation of *kdp* gene expression in *E. coli* and *H. salinarum* likely resides in the different physiological context of the cell (Fig. S6).

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