Construction and characterization of a gradually inducible expression vector for *Halobacterium salinarum* based on the *kdp* promoter

Dorthe Kixmüller and Jörg-Christian Greie*

Universität Osnabrück, Fachbereich Biologie/Chemie, D-49069 Osnabrück, Germany

* To whom correspondence should be addressed.

Jörg-Christian Greie
Universität Osnabrück
Fachbereich Biologie/Chemie
Barbarastrasse 11
49069 Osnabrück
Germany
Phone: +49 541 969 2809
Fax: +49 541 969 2870
E-mail: greie@biologie.uni-osnabrueck.de

Running title: An inducible expression vector for *H. salinarum*
Abstract

Gradually inducible expression vectors which are governed by variations of growth conditions are powerful tools for gene expression of conditionally lethal mutants. Furthermore, controlled expression allows monitoring of overproduction of proteins at various stages in their expressing hosts. For *Halobacterium salinarum*, which is often used as a paradigm for halophilic Archaea, such an inducible expression system is not available to date. Here we show that the *kdp* promoter (*P*<sub>kdp</sub>), which facilitates gene expression upon K<sup>+</sup> limitation, can be used to establish such a system for molecular applications. *P*<sub>kdp</sub> features a rather high expression rate with an approximately 50-fold increase that can be easily varied by K<sup>+</sup> concentrations in the growth medium. Besides the construction of an expression vector, our work describes the characterization of expression patterns and, thus, offers a gradually inducible expression system to the scientific community.
Introduction

Research on the archaea as the third domain of life has become an increasing focus of attention since they combine bacterial and eukaryotic characteristics. The archaeal core metabolism resembles that of bacteria, whereas their information processing systems share eukaryotic properties (1). The fact that generic molecular principles like transcription (3, 9) and DNA replication (7) also correspond to that of the eukaryotic organisms additionally increases research interest, since the archaeal systems are more basic and, thus, easier accessible than their rather complex counterparts in eukaryotes. However, scientific research on archaea is lagging with respect to that in bacteria and eukaryotes since corresponding analytical tools especially at the level of molecular biology are only poorly available (13). Although generic tools like transformation protocols, shuttle vectors, multiple resistance markers and reporter genes are yet available for at least some halophilic as well as for thermophilic and methanogenic archaea (6, 14), gene expression systems with tightly regulated promoters are actually missing. The controlled expression of target genes is an essential feature to monitor the overproduction of proteins. Besides mere protein overproduction, a gradual gene expression also enables more sophisticated applications like the synthesis of dosage-dependent toxic proteins or of inactive mutant forms of essential members of cellular pathways in a wild type background. Another promising application is the upcoming analyses of effects of antisense sequences in gene regulation. Although corresponding expression systems are already well-established within the domains Bacteria and Eukarya, several attempts to develop an adequate inducible expression system for the Archaea failed for a long period of time (e.g. 14). Only recently, such systems have been developed at least for Sulfolobus solfataricus and Haloferax volcanii (2, 13, 17) but are still lacking in case of Halobacterium salinarum.
In our previous studies we could show that expression of the \textit{kdpFABCQ} operon, which encodes the K$^+$ uptake system KdpFABC in \textit{H. salinarum}, was strictly dependent on the K$^+$ concentration in the growth medium (11, 18). At extracellular K$^+$ concentrations below 20 mM the \textit{kdpFABCQ} operon is moderately expressed, whereas expression significantly increased below 250 µM with the highest expression level present at 20 µM external K$^+$. At this concentration the cells ceased to grow due to the affinity limit of the KdpFABC complex for K$^+$. More detailed studies of the \textit{kdp} promoter (\textit{P\textsubscript{kdp}}) revealed an operator sequence upstream of the basal transcription elements as well as KdpQ acting as co-regulator stimulating expression under inducing conditions (11, 18).

Although the particular interplay of these regulatory components is still under investigation, further regulatory components within the \textit{kdpFABCQ} coding region are clearly absent (11). Plasmid-encoded \textit{kdp} operon expression from \textit{P\textsubscript{kdp}} resulted in an induction ratio of ~50 for non-induced vs. induced activity (11). These findings readily render \textit{P\textsubscript{kdp}} to be a promising candidate in order to establish a system in which halobacterial gene expression can be controlled by the simple manipulation of growth conditions (i. e. KCl concentrations). Since the transcriptional regulatory elements of \textit{P\textsubscript{kdp}} have already been studied in detail (11), this allows a deliberate construction of a versatile expression system.

This work deals with the construction and characterization of a replicating vector containing the promoter of the \textit{kdpFABCQ} operon, thus enabling the controlled inducible expression of target genes in \textit{H. salinarum}. Since the correlation between gene expression and subsequent protein synthesis varies over a broad range among different genes, a statement on the general applicability of an expression system with respect to protein synthesis is difficult to achieve. Thus, this paper focuses on gene expression (i. e. transcript analyses), which is solely dependent on the promoter and is usually not affected by the subsequent translational processes.
Materials and Methods

Strains, plasmids and growth conditions

The haloarchaeal strains used in this study are *H. salinarum* R1 (15), *H. salinarum* R1 ΔkdpFABC (18) and *H. salinarum* R1 ΔkdpFABCQ (18). Plasmids and primers are listed in Tables S1 and S2.

*H. salinarum* R1 strains were grown in rich media as described for *Halobacterium* sp. NRC-1 (American Type Culture Collection medium 2185). Cultivation of *H. salinarum* strains in media with different initial K⁺ concentrations was carried out as described previously (18). *Halobacterium* cultures were grown aerobically under illumination at 37°C. If required, media were supplemented with 20 μg/ml of Simvastatin (Ratiopharm). Sampling of *H. salinarum* cultures grown under *P*kpdp-inducing (K⁺ limitation; 3 mM initial KCl) and non-inducing conditions (non-limiting K⁺ concentrations; 100 mM initial KCl) was performed as previously described (11).

Transformation of *H. salinarum* was carried out by use of polyethylene glycol 600 (5). For *bgaH*-encoded β-galactosidase activity assays and *kdpFABCQ* expression studies by real-time RT-PCR, corresponding plasmids were transformed into *H. salinarum* R1 ΔkdpFABC. Plasmids for *kdpFABCQ* expression studies by real-time RT-PCR were additionally transformed in *H. salinarum* R1 ΔkdpFABCQ.

Construction of pKIX

Plasmid pMKK100 was restricted via ClaI and SapI in order to obtain a DNA fragment comprising the resistance genes and the ColE1 origin. A fragment carrying a multiple cloning site (MCS) was obtained via PCR by use of Phusion® High-Fidelity DNA Polymerase (Finnzymes) on pBAD24 as template. ClaI and SapI restriction sites flanking the MCS were introduced via
primers 1 and 2. Restriction and ligation of the pMKK100 and the MCS fragment resulted in plasmid pKIXpre1. The pHK2 origin of replication was derived from plasmid pMDS20 by restriction with AvrII and KpnI. The resulting pHK2 ori fragment was further restricted via SacI. To clone the pHK2 fragment into pKIXpre1, a two-step PCR was performed on this plasmid by use of primers 3 and 4 together with primers 5 and 6 in order to introduce the AvrII and SacI restriction sites. Restriction with AvrII and SacI and subsequent ligation with the pHK2 fragment resulted in pKIXpre2. The kdp promoter was amplified via PCR on H. salinarum R1 genomic DNA, and restriction sites EcoRI and NcoI flanking Pkdp were inserted via primers 7 and 8. By use of EcoRI and NcoI restriction/ligation, the Pkdp fragment was inserted into the MCS of pKIXpre2, resulting in pKIXPkdp. Finally, the kdp terminator (11) was amplified by PCR on H. salinarum R1 genomic DNA via primers 9 and 10, thereby introducing HindIII and AvrII restriction sites flanking the terminator, and subsequently ligated into properly digested pKIXPkdp, which resulted in plasmid pKIX. For expression studies of bgaH under the control of Pkdp, the bgaH gene was amplified from pMKK100 as template via primers 11 and 12, which also introduced NcoI and HindIII restriction sites. By use of these restriction sites it was possible to put bgaH under control of Pkdp, resulting in plasmid pKIX_bgaH. To introduce the entire kdpFABCQ operon (including Pkdp and the kdp terminator) into pKIX, the operon was restricted via NcoI and HindIII from a pBAD24kdpFABCQ subcloning vector (11, 18) and subsequently cloned into the MCS of pKIXpre1, resulting in plasmid pKIX_kdpFABCQ. For further details on pKIX construction and plasmid sequence, please refer to the supplemental material (Fig S1).

Construction of mutants

Substitutions in Pkdp fused to bgaH were generated on a plasmid with a -42 bp truncated promoter (pDSK12). Mutations were introduced by swapping purines to pyrimidines and vice
versa. These substitutions were generated by two-step PCR via Phusion® High-Fidelity DNA Polymerase with pDSK12 as template and the outer primers 13 and 14. The inner primers 15 and 16 were used to generate a nucleotide exchange between the transcription start site of Pkdp and the start codon, whereas the inner primers 17 and 18 as well as the inner primers 19 and 20 were used to generate exchanges in the region -8 to -11 and -17 to -20, respectively. Restriction of the PCR fragments and pHJS4 with EcoRI and PstI with subsequent ligation resulted in plasmids pDSK20-22.

To investigate the combined influence of substitutions in regions -8 to -11 and -17 to -20 on kdpFABCQ expression by real-time RT-PCR analyses, both sets of mutations were introduced via two-step PCR on plasmid pHJS1 by use of the outer primers 21 and 22 together with inner primers 23 and 24. The resulting PCR product was restricted via NcoI and KpnI and subcloned into pBAD24_kdpFABCQ. Mutation-containing fragments with the entire kdpFABCQ operon could then be cloned into pHJS4 via NcoI and HindIII, which resulted in pDSK23.

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

Total RNA was isolated via the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions, except for the use of lysozyme in TE buffer. Residual DNA contaminations were eliminated by DNase I digestion on 140 ng RNA. Reverse transcription was performed on 70 ng of total RNA with random hexamer primers by use of the First Strand cDNA Synthesis Kit (Fermentas). Real-time RT-PCR analyses to quantify kdpFABCQ expression were performed by use of primers 25 and 26 annealing in kdpC and kdpQ, respectively. Quantitative analyses of bgaH transcript levels were carried out by use of primers 27 and 28. Primers 29 and 30 targeted at the housekeeping gene rpoA1 in H. salinarum R1 were used to normalize the calculated Ct values. Basically, real-time RT-PCR was carried out as described in (18).
β-galactosidase assays

β-galactosidase activity in cell lysates was measured using the ONPG assay as previously described (8, 11).

Determination of K⁺ concentrations

Residual K⁺ concentrations in the growth medium were analyzed by flame emission photometry as described previously (11).

Results

Prerequisites for an inducible expression system based on Pkdp

Since haloarchaea and especially H. salinarum have rather long generation times (10, 12), a rapid and convenient cloning and isolation of DNA can, however, be realized by the use of an Escherichia coli – H. salinarum shuttle vector. The expression vector pKIX comprises a ColE1 origin of replication and the bla (β-lactamase) gene as selectable marker in E. coli. For cloning and manipulation in Halobacterium, the plasmid features a pHK2 origin of replication (copy number of about eight) together with the resistance marker hmg (HMG-CoA reductase) for selection on mevinolin and/or simvastatin.

The basic features for replication and selection are completed by the principal component Pkdp, which was inserted into the multiple cloning site 3 (MCS 3) of the pBAD vector series. To prevent a potential read-through effect emanating from Pkdp, the kdp terminator (11) was also applied (Fig. 1).

The transcription start site defined by Pkdp is located 4 bp upstream of the kdpF start codon, thereby resulting in a leaderless transcript (11). For convenient cloning of target genes, the 5’-
CCATGG-3' *NcoI* sequence was inserted into the native start codon. Therefore, two base pairs upstream the start codon had to be exchanged. To test whether these nucleotides have an influence on the basal transcription initiation and, thus, on transcription efficiency or whether this region interferes with translational performance, the three base pairs located in between the transcription start site and the native start codon were swapped from purine to pyrimidine and vice versa in a truncated -42 bp *Pkdp* (Fig. 2a). This version of the *kdp* promoter is deleted in the operator (located from -55 bp to -74 bp) and is translationally fused to the halophilic β-galactosidase gene *bgaH* (8, 14) in a background of an integrative vector. Since the operator sequence is deleted, the non-induced expression (100 mM initial K⁺ in the medium) is increased (11) (Fig. 2b). However, no influence on β-galactosidase activity due to the purine/pyrimidine exchanges could be determined. Therefore, mutated nucleotides in the region between the transcription start site and the start codon have neither an influence on transcription efficiency nor an effect on the translation process.

**Controlled expression of *bgaH* governed by *Pkdp* in pKIX**

In previous experiments we determined the β-galactosidase activities of a *Pkdp::bgaH* fusion over a broad range of K⁺ concentrations from 3 to 100 mM, which demonstrated that the level of *kdp* operon expression is clearly dependent on medium K⁺ concentration (Fig. S2). To investigate gene expression from *Pkdp* mediated by pKIX, we selected three different initial K⁺ concentrations, which showed either maximal (3 mM initial KCl), approximately half-maximal (5 mM initial KCl) or no (100 mM initial KCl) expression. These concentrations have already successfully been used to investigate the expression patterns of the natural *kdpFABCQ* genes (18).
To determine expression levels from pKIX with a “foreign” gene other than the natural $kdpFABCQ$ operon, cells of $H. salinarum$ R1 $\Delta kdpFABC$ were transformed with pKIX$_{bgaH}$ and cultured in media containing 3 mM, 5 mM and 100 mM initial KCl (Fig. 3). As expected, the culture grown with 3 mM KCl (grey diamonds) entered the stationary growth phase earlier than the cultures supplemented with 5 mM (grey squares) and 100 mM KCl (black triangles), which was well in accord with previous data (18). At this time point the 3 mM culture ceased to grow due to K$^+$ limitation. This effect is even more pronounced with respect to our previous data since the $kdp$ genes are absent. The residual medium K$^+$ concentration was determined to be 60 $\mu$M, which is known to be the minimal concentration for K$^+$ uptake of $H. salinarum$ without a functional Kdp complex (18). However, cultures grown with 5 mM and 100 mM KCl did not comprise a growth-restricting K$^+$ depletion. Growth of the 3 mM KCl culture could be stimulated by the addition of 100 mM KCl in the late exponential growth phase (black circles), thus demonstrating that growth restriction is due to potassium limitation.

In parallel, samples were taken to follow the pKIX-dependent $bgaH$ expression quantitatively via real-time RT-PCR (Fig. 4). As expected, the culture grown under non-inducing K$^+$ concentrations of 100 mM KCl exhibited a constant constitutive level of $bgaH$ expression during growth, which was very low but, nevertheless, detectable. In contrast, the 3 mM KCl culture exhibited a slight increase in $bgaH$ expression already in the early exponential growth phase at an OD$_{600}$ of 0.5, which is due to upcoming K$^+$ limitation. Expression further increased in the run of K$^+$ depletion during the late exponential growth phase and reached its maximum at the lowest medium K$^+$ concentration of 60 $\mu$M in the early stationary phase. At that point, $bgaH$ expression was almost 50-fold induced with respect to the low constitutive level of expression obtained in the presence of non-inducing 100 mM KCl in the medium. This value is in accord with that determined previously for the natural $kdpFABCQ$ genes (11). In the late stationary growth phase, expression...
was found to be significantly down-regulated. The culture grown with 5 mM initial KCl 
exhibited a similar expression pattern but to an overall lower level, thereby indicating less severe 
K$^+$ depletion due to the higher initial KCl concentration. This $bgaH$ expression pattern again 
corresponded well with our previous studies on the natural $kdpFABCQ$ operon expression (18). 
However, it should be denoted at this point that the natural non-induced constitutive expression 
rate of chromosomally encoded $kdpFABCQ$ is generally lower which, in turn, leads to higher 
induction ratios (18).

Whereas $P_{kdp}$-governed $bgaH$ expression is clearly stimulated by K$^+$ depletion, it is repressed by 
the presence of potassium. This could be demonstrated by the subsequent addition of 100 mM 
KCl to the 3 mM KCl culture in the late exponential growth phase. A decrease in $bgaH$ 
expression following the addition of KCl could already be observed after 1.5 hours. A total 
downregulation with expression levels comparable to that of the culture with 100 mM initial KCl 
could be observed in the early stationary phase, where expression normally reaches its maximum. 
These experiments demonstrate that the expression of a “foreign” gene from $P_{kdp}$ in pKIX 
correlates well with the potassium concentrations in the growth medium. Adjusting the KCl 
concentration allows for easy manipulation of expression levels in both directions, thereby also 
enabling a rather rapid blocking of gene expression by the addition of large amounts of 
potassium.

**Improvement of pKIX mediated expression levels**

In our previous studies we could show that KdpQ acts as a co-regulator by stimulating $kdp$ 
operon expression under inducing conditions (11, 18). Thus, the additional presence of the $kdpQ$ 
gene on pKIX together with the chromosomally encoded $kdpQ$ should further enhance gene 
expression from pKIX. In order to test this hypothesis, $kdpFABCQ$ expression from pKIX
(pKIX_kdpFABCQ) was monitored in *H. salinarum* R1 ΔkdpFABC, which still comprises chromosomally encoded KdpQ under control of Pkdp.

To keep the results comparable to that of pKIX-derived bgaH expression, cell growth, sampling points and subsequent quantitative real-time RT-PCR analyses were set up correspondingly (Fig. 5). As expected, both the 3 mM KCl culture and the 5 mM KCl culture showed a clear increase in the overall kdpFABCQ transcript level at every point of time compared to the bgaH expression levels obtained with only the chromosomally encoded KdpQ (compare Fig. 4). The enhanced expression, however, did not notably affect expression patterns. Since the low constitutive level of kdpFABCQ expression obtained in the presence of 100 mM initial KCl was also not affected, an elevated induction ratio of >400 could be derived from this study, which is due to the additional presence of *kdpQ* on pKIX. Thus, gene expression can clearly be boosted by incorporating *kdpQ* as transcriptional activator on the expression plasmid.

In our previous studies we investigated the transcriptional regulation of *kdp* operon expression by use of an integrative vector comprising various mutations in Pkdp between the TATA box and the transcription start site (11). Some of these mutations revealed to have a positive effect on the induced transcription level and were now investigated for their potential to further boost Pkdp-governed gene expression. Purine/pyrimidine exchanges were generated in the region -8 to -11 bp and -17 to -20 bp (Fig. 6a) within a truncated promoter. This mutated -42 bp Pkdp was then fused to bgaH and assayed for bgaH expression on the protein level (Fig. 6b). The purine/pyrimidine swaps in both regions resulted in an increase of the induced (3 mM KCl) expression by a factor of 2.5 compared to the non-modified -42 bp promoter. However, exchanges in the -8 to -11 region also resulted in a slight increase in expression even under non-inducing conditions. The overall rather high non-induced expression level is due to the deleted operator sequence in the truncated promoter background. For further analyses, both sets of mutations were generated in a -206 bp...
full-length *kdp* promoter construct in the same integrative vector background and *kdpFABCQ* expression was analyzed in *H. salinarum* R1 Δ*kdpFABCQ* on the transcript level by real-time RT-PCR (Fig. 6c). Due to the presence of the operator within the full-length *Pkdp*, non-induced expression is barely detectable, thus yielding higher overall induction ratios. However, whereas the wild-type *Pkdp* achieved an induction ratio of about 50, the concomitant presence of the purine/pyrimidine swaps in regions -8 to -11 bp and -17 to -20 bp resulted in an almost four times higher induction ratio of about 190. The swaps did not effect the non-induced expression, thereby demonstrating that the promoter is still tightly regulated. 

Taken together, expression levels from *Pkdp* are dosage dependent with respect to KdpQ and can be increased by specific mutations within the promoter. Thus, pKIX-derived expression rates could further be boosted by the incorporation of these features.

**Discussion**

Our previous studies on the promoter of the *kdpFABCQ* operon of *H. salinarum* revealed a clear dependency of operon expression with respect to the K\(^+\) concentration in the medium. By use of this key feature of *Pkdp*, expression can easily be modulated by simple variations of the initial K\(^+\) concentration or by the addition of KCl. The construction of the replicating vector pKIX made *Pkdp* available for the controlled expression of target genes. The plasmid pKIX enables *Pkdp*-promoted K\(^+\)-dependent gene expression with rather high induction ratios of up to about 50-fold and, thus, has the potential to serve as a generic inducible expression system for *Halobacterium*. However, due to its nature of modulation, pKIX is not applicable as gradually inducible expression system under balanced growth conditions. The window of maximum induction under...
K⁺-limiting growth conditions is transient and occurs as cells enter the stationary phase due to K⁺ depletion.

Patterns of Pkdp-promoted expression of the β-galactosidase gene bgaH were comparable to that of the natural kdpFABCQ genes, which already argues for constant Pkdp-promoted expression patterns independent of the cloned target gene. The real-time RT-PCR analyses revealed that pKIX-mediated expression is on a low constitutive level even under non-inducing conditions, which is in accord with the low basal transcript level observed with an integrative vector (11). This observation implies a less tight repression of Pkdp under non-inducing conditions. However, in our previous studies we could show that this low constitutive expression has no detectable effect on the β-galactosidase protein level, since an integrative vector encoding bgaH translationally fused to the full-length Pkdp resulted in enzyme activities under non-inducing conditions (100 mM KCl) which were not above the background level (11). Nevertheless, although an effect on the level of β-galactosidase activity could not be detected, this residual constitutive activity should be taken into account in more deliberate zero expression studies.

The results gained from this study revealed that a significant increase in both the expression and induction ratio could be observed in case of the concomitant presence of kdpQ on both plasmid and chromosome. This KdpQ-related enhancing effect could further be used for the expression of target genes even at higher initial K⁺ concentrations in order to circumvent extreme K⁺ limitation, which forces the cells to cease growth. Thus, the introduction of kdpQ to the pKIX vector is a worthwhile approach for further investigations.

A similar effect could be obtained by the introduction of the purine/pyrimidine swaps in pKIX. The enhanced Pkdp-derived expression under inducing conditions could further contribute to higher expression levels in general. This effect most likely resides in an improved recruitment of the basal transcription machinery. The analyses of numerous haloarchaeal promoters revealed a
“WW” element at positions -10 and -11, which has been suggested to represent a novel basal promoter element (4). Possible candidates binding to this motif are the basal transcription factor B (TFB) and the RNA polymerase (16). By the introduction of the purine/pyrimidine swaps in region -8 to -11 an “AA” sequence was generated at this position. These inserted “WW” nucleotides might optimize \( P_{kdp} \) for an enhanced expression by enhancing the binding affinity of the transcription initiation components mentioned above. Accordingly, substitutions performed in the region -17 to -20 could also affect the binding affinity of TFB and/or the RNA polymerase (16).

The conclusions drawn from our present study strongly indicate that pKIX might function as an adequate inducible expression system. So far, halophilic proteins could only be expressed in \( H. volcanii \) by use of a recently introduced system for the conditional overexpression of genes (2). Thus, if expression of a target gene is not limited to \( H. salinarum \), this system already constitutes a well-qualified tool for inducible gene expression of halophilic proteins. However, the corresponding expression vectors are based on the tryptophan-inducible \( P_{tnaA} \) (tryptophanase) promoter and are designed for a restrictive application in combination with corresponding \( H. volcanii \) strains. Thus, these vectors are not applicable for \( H. salinarum \). Furthermore, \( P_{tnaA} \) activity and application has not yet been shown in \( H. salinarum \).

**Acknowledgements**

This work was supported by the Deutsche Forschungsgemeinschaft (grant GR2698/1-1).
References


Figure legends

FIG. 1. Map of the inducible expression vector pKIX. The multiple cloning site (MCS) and the inserted kdp promoter (Pkdp) with the basal transcription initiation elements (TATA, BRE and INR) are shown in detail. The transcription start site in Pkdp is denoted by an arrow. Unique restriction sites suitable for the cloning of target genes within the MCS are highlighted in light grey.

FIG. 2. (a) Sequence of Pkdp between the TATA box and the transcription start site (arrow). Exchanged nucleotides (ex) are denoted. (b) β-galactosidase activities of transcriptional fusions of bgaH to a truncated -42 bp Pkdp (-42 Pkdp) and to -42bp Pkdp with the nucleotide exchanges shown in (a) (-42 Pkdp ex). Measurements were done in triplicate with H. salinarum R1 ΔkdpFABC cultures grown under inducing and non-inducing conditions (3 and 100 mM initial K⁺, respectively).

FIG. 3. Growth of H. salinarum R1 ΔkdpFABC transformed with pKIX_bgaH in medium supplemented with 3 mM (diamonds), 5 mM (squares) and 100 mM (triangles) of KCl. Another 3 mM KCl culture (circles) was supplied with 100 mM KCl in the late exponential growth phase (arrow) directly upon sampling for subsequent real-time RT-PCR analyses (see Fig. 5). Samples were collected at the times indicated by arrows in the early exponential (early exp.), the late exponential (late exp.), the early stationary (early stat.) and the late stationary (late stat.) growth phase. In case of the 3 mM KCl culture supplied with 100 mM KCl, an additional sample was taken 1.5 h subsequent to KCl addition.
FIG. 4. Normalized transcript levels of *bgaH* expressed from pKIX_ *bgaH* in *H. salinarum* R1 Δ*kdpFABC* with varying initial K⁺ concentrations in the medium (3 mM, 5 mM and 100 mM KCl). Samples were taken from cultures as described in Fig. 4. All samples were assayed in triplicate by real-time RT-PCR analysis.

FIG. 5. Normalized transcript levels of *kdpFABCQ* expressed in *H. salinarum* R1 Δ*kdpFABC* from pKIX_kdp with varying initial K⁺ concentrations in the medium (3 mM, 5 mM and 100 mM KCl). Sampling was done as described in Fig. 5. All samples were assayed in triplicate by real-time RT-PCR analysis.

FIG. 6. (a) Sequence of P_kdp* between the TATA box and the transcription start site (arrow). Mutations introduced in regions -17 to -20 (-17_-20) and -8 to -11 (-8_-11) are denoted. (b) β-galactosidase activities of *H. salinarum* R1 Δ*kdpFABC* encoding P_kdp::*bgaH* fusions with a truncated -42 bp P_kdp* (-42). -17_-20 and -8_-11 indicate effects of the exchanges shown in (a). Samples were taken from cultures grown under inducing and non-inducing conditions (3 and 100 mM KCl, respectively) and analyzed in triplicate. (c) Normalized expression levels of *kdpFABCQ* expressed in *H. salinarum* R1 Δ*kdpFABCQ* from the full length *kdp* promoter (-206) and from the promoter comprising both sets of mutations depicted in (a) (-206 ex). Measurements were performed in triplicate by real-time RT-PCR analysis.