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Construction and Characterization of a Gradually Inducible Expression Vector for *Halobacterium salinarum*, Based on the *kdp* Promoter

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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*kdp*), which facilitates gene expression upon K^+ limitation, can be used to establish such a system for molecular applications. P*kdp* features a rather high expression rate, with an approximately 50-fold increase that can be easily varied by K^+ 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.

Research on the archaea as the third domain of life has become an increasing focus of attention because archaea 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 those of the eukaryotic organisms additionally increases research interest, since the archaeal systems are more basic and, thus, more easily accessible than their rather complex counterparts in eukaryotes. However, scientific research on archaea is lagging with respect to that on 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 available for at least some halophilic as well as 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 analysis 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). Recently, such systems have been developed at least for *Sulfolobus solfataricus* and *Haloferax volcanii* (2, 13, 17), but they are still lacking in the case of *Halobacterium salinarum*.

In our previous studies, we showed that expression of the *kdpFABCQ* operon, which encodes the K^+ uptake system KdpFABC in *H. salinarum*, was strictly dependent on the K^+ concentration in the growth medium (11, 18). At extracellular K^+ concentrations below 20 mM, the *kdpFABCQ* operon is moderately expressed, whereas expression significantly increased with concentrations below 250 μM, with the highest expression level present at a 20 μM concentration of 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 *kdp* promoter (P*kdp*) revealed an operator sequence upstream of the basal transcription elements as well as KdpQ acting as a coregulator stimulating expression under inducing conditions (11, 18). Although the particular interplay of these regulatory components is still under investigation, further regulatory components within the *kdpFABCQ* coding region are clearly absent (11). Plasmid-encoded *kdp* operon expression from P*kdp* resulted in an induction ratio of ~50 for noninduced activity versus induced activity (11). These findings readily render P*kdp* a promising candidate in the establishment of 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 P*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 *kdpFABCQ* operon, thus enabling the controlled inducible expression of target genes in *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.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The haloarchaeal strains used in this study are *H. salinarum* R1 (15), *H. salinarum* R1 Δ*kdpFABC* (18), and *H. salinarum* R1 Δ*kdpFABCQ* (18). Plasmids and primers are listed in Tables S1 and S2 in the supplemental material.

H. salinarum R1 strains were grown in rich medium as described for *Halobacterium* sp. NRC-1 (ATCC 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, the medium was supplemented with 20 μg/ml of simvastatin (Ratiopharm). Sampling of *H. salinarum* cultures grown under P*kdp*-inducing (K^+ limitation; 3 mM initial KCl) and noninducing conditions (nonlimiting 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 reverse transcriptase PCR (RT-PCR), corresponding plasmids were transformed into *H. salinarum* R1 Δ*kdpFABC*. Plasmids for *kdpFABCQ* expression studies by real-time RT-PCR were additionally transformed in *H. salinarum* R1 Δ*kdpFABCQ*.

Construction of pKIX. The 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 a template. ClaI and SapI restriction sites flanking the MCS were introduced via primers 1 and 2. Restriction and ligation of 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 P*kdp* were inserted via primers 7 and 8. By use of EcoRI and NcoI restriction/ligation, the P*kdp* fragment was inserted into the MCS of pKIXpre2, resulting in pKIXP*kdp*. 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 pKIXP*kdp*, which resulted in plasmid pKIX. For expression studies of *bgaH* under the control of P*kdp*, the *bgaH* gene was amplified from pMKK100 as a 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 the control of P*kdp*, resulting in plasmid pKIX_*bgaH*. To introduce the entire *kdpFABCQ* operon (including P*kdp* and the *kdp* terminator) into pKIX, the operon was restricted via NcoI and HindIII from a pBAD24*kdpFABCQ* subcloning vector (11, 18) and subsequently cloned into the MCS of pKIXpre1, resulting in plasmid pKIX_*kdpFABCQ*. See Fig. S1 in the supplemental material for further details on pKIX construction and plasmid sequence.

Construction of mutants. Substitutions in P*kdp* fused to *bgaH* were generated on a plasmid with a truncated P*kdp* promoter consisting of bp −1 to −42 (−42-bp P*kdp*) (pDSK12). Mutations were introduced by swapping purines for pyrimidines and vice versa. These substitutions were generated by two-step PCR via Phusion high-fidelity DNA polymerase with pDSK12 as the 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 P*kdp* 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 regions of bp −8 to −11 and bp −17 to −20, respectively. Restriction of the PCR fragments and pHJS4 with EcoRI and PstI and subsequent ligation resulted in plasmids pDSK20 to pDSK22.

To investigate the combined influence of substitutions in the regions of bp −8 to −11 and bp −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 the 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 were then cloned into pHJS4 via NcoI and HindIII, a process which resulted in pDSK23.

RNA extraction, cDNA synthesis, and real-time RT-PCR. Total RNA was isolated via the RNeasy minikit (Qiagen) according to the manufacturer's instructions except for the use of lysozyme in Tris-EDTA (TE; pH 8) 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 threshold cycle (C_T) values. Basically, real-time RT-PCR was carried out as described in reference 18.

β-Galactosidase assays. β-Galactosidase activity in cell lysates was measured using the *o*-nitrophenyl-β-D-galactopyranoside (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 P*kdp*. Since haloarchaea and especially *H. salinarum* have rather long generation times (10, 12), a rapid and convenient cloning and isolation of DNA can 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 a selectable marker in *E. coli*. For cloning and manipulation in *Halobacterium*, the plasmid features a pHK2 origin of replication (copy number of about 8) together with the resistance marker *hmg* (3-hydroxy-3-methylglutaryl-coenzyme A [HMG-CoA] reductase) for selection on mevinolin and/or simvastatin.

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

The transcription start site defined by P*kdp* 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′-CC ATGG-3′ NcoI sequence was inserted into the native start codon. Therefore, 2 base pairs upstream of 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 3 base pairs located between the transcription start site and the native start codon were switched from purine to pyrimidine and vice versa in a truncated −42-bp P*kdp* (Fig. 2a). This version of the *kdp* promoter is deleted in the operator (located between bp −55 and −74) 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 noninduced expression (100 mM initial K^+ in the medium) is increased (11) (Fig. 2b). However, no influence on β-galactosidase

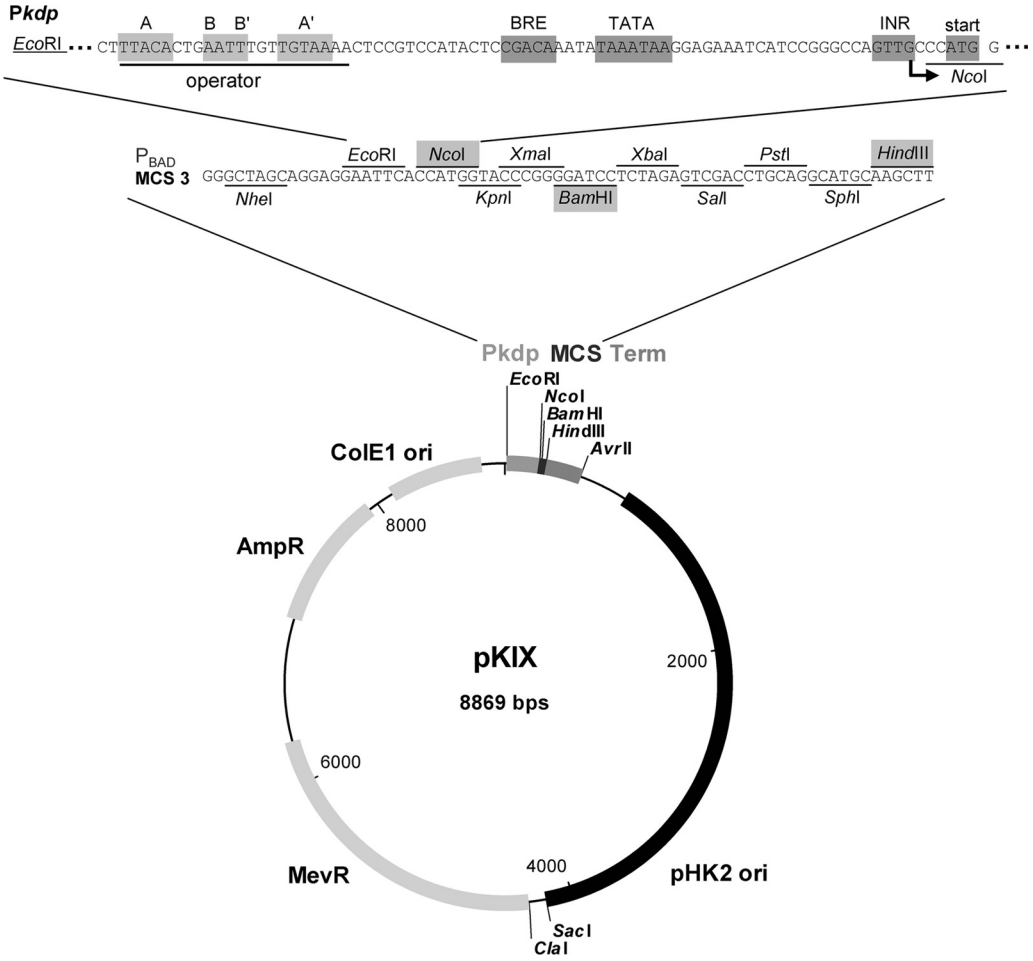

FIG 1 Map of the inducible expression vector pKIX. The multiple-cloning site (MCS) and the inserted *kdp* promoter (P*kdp*) with the basal transcription initiation elements (TATA box [TATA], transcription factor B recognition element [BRE], and initiation region [INR]) are shown in detail. The transcription start site in P*kdp* is denoted by an arrow. Unique restriction sites suitable for the cloning of target genes within the MCS are highlighted in light gray.

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 P*kdp* in pKIX. In previous experiments, we determined the β-galactosidase activities of a P*kdp*::*bgaH* fusion over a broad range of K^+ concentrations from 3 to 100 mM and demonstrated that the level of *kdp* operon expression is clearly dependent on the K^+ concentration of the medium (see Fig. S2 in the supplemental material). To investigate gene expression from P*kdp* mediated by pKIX, we selected three different initial K^+ concentrations which showed either maximal (3 mM initial KCl), approximately half of the maximal (5 mM initial KCl), or no (100 mM initial KCl) expression. These concentrations have already been successfully 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 genes of the natural *kdpFABCQ* operon, cells of *H. salinarum* R1 Δ*kdpFABC* were transformed with pKIX_*bgaH* and cultured in medium containing 3 mM, 5 mM, or 100 mM initial KCl (Fig. 3). As expected, the culture grown with 3 mM KCl (diamonds) entered the stationary growth phase earlier than the cultures supplemented with 5 mM (squares) and 100 mM KCl (triangles), a result 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 K^+ concentration in the medium was determined to be 60 μM, 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 (circles), thus demonstrating that growth restriction is due to potassium limitation.

Samples were taken in parallel to follow the pKIX-dependent *bgaH* expression quantitatively via real-time RT-PCR (Fig. 4). As expected, the culture grown under noninducing 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 already exhibited a slight increase in *bgaH* expression in the early exponential growth phase at an optical density at 600 nm (OD_{600}) of 0.5, a

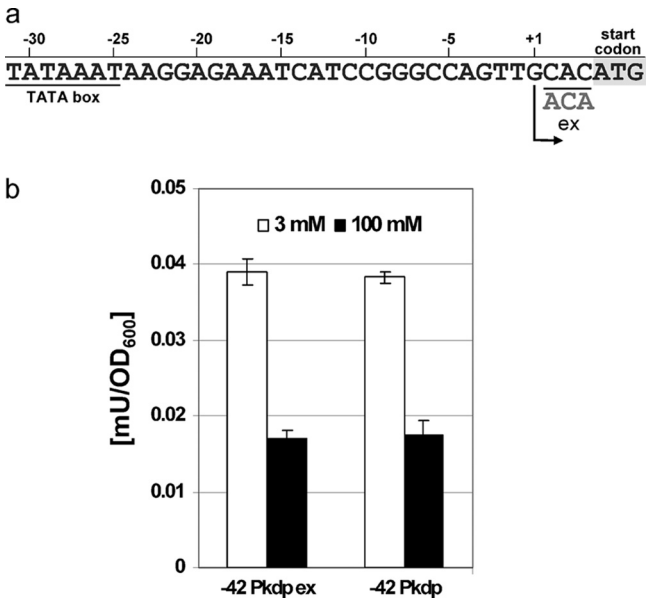

FIG 2 (a) Sequence of P*kdp* between the TATA box and the transcription start site (arrow). Exchanged nucleotides (ex) are denoted. (b) β-Galactosidase activities of transcriptional fusions of *bgaH* to a truncated −42-bp P*kdp* (−42 Pkdp) and to the −42-bp P*kdp* with the nucleotide exchanges (−42 Pkdp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

result 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 K^+ concentration of 60 μM in the early stationary phase. At that point, *bgaH* expression was induced almost 50-fold with respect to the low constitutive level of expression obtained in the presence of non-

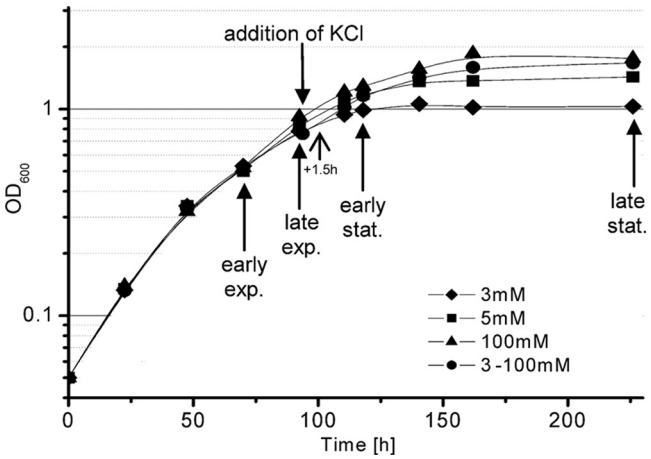

FIG 3 Growth of *H. salinarum* R1 Δ*kdpFABC* transformed with pKIX_*bgaH* in medium supplemented with 3 mM, 5 mM, or 100 mM KCl. Another 3 mM KCl culture was supplied with 100 mM KCl in the late exponential growth phase (arrow) directly upon sampling for subsequent real-time RT-PCR analyses (see Fig. 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 phases. In the case of the 3 mM KCl culture supplied with 100 mM KCl, an additional sample was taken 1.5 h following KCl addition.

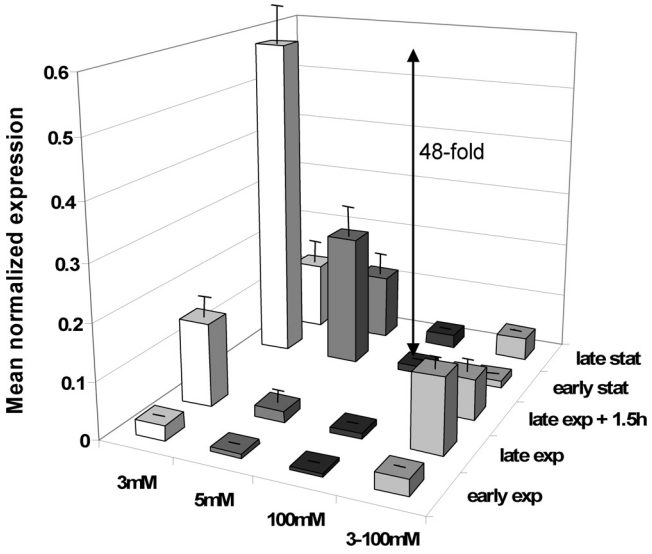

FIG 4 Normalized transcript levels of *bgaH* expressed from pKIX_*bgaH* in *H. salinarum* R1 Δ*kdpFABC* with various initial K^+ concentrations in the medium (3 mM, 5 mM, and 100 mM KCl). Samples were taken from cultures as described in the legend to Fig. 3. All samples were assayed in triplicate by real-time RT-PCR analysis.

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 downregulated. 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 noninduced 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 was 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 h. A total downregulation with expression levels comparable to those of the culture with 100 mM initial KCl was observed in the early stationary phase, in which 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 showed that KdpQ acts as a coregulator 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

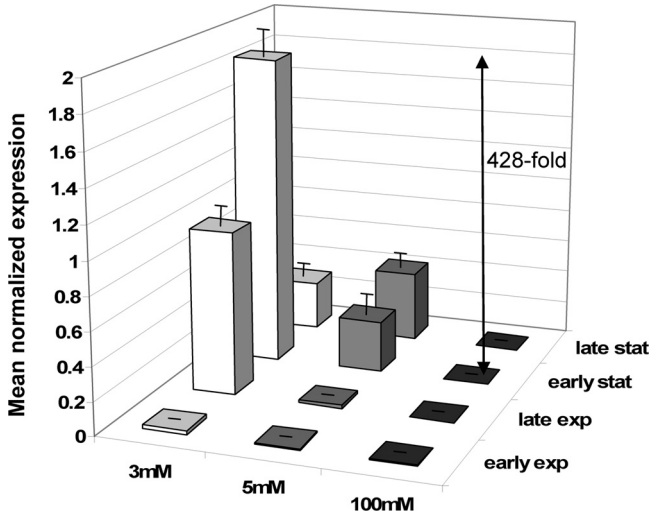

FIG 5 Normalized transcript levels of *kdpFABCQ* expressed in *H. salinarum* R1 Δ*kdpFABC* from pKIX_*kdp* with various initial K^+ concentrations in the medium (3 mM, 5 mM, and 100 mM KCl). Sampling was done as described in the legend to Fig. 3. All samples were assayed in triplicate by real-time RT-PCR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

monitored in *H. salinarum* R1 Δ*kdpFABC*, which still comprises chromosomally encoded KdpQ under the control of P*kdp*.

To keep the results comparable to those 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 in time compared to the *bgaH* expression levels obtained with only the chromosomally encoded KdpQ (compare to 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, which is due to the additional presence of *kdpQ* on pKIX, could be derived from this study. Thus, gene expression can clearly be boosted by incorporating *kdpQ* as a 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 P*kdp* between the TATA box and the transcription start site (11). Some of these mutations were revealed to have a positive effect on the induced transcription level and were now investigated for their potential to further boost P*kdp*-governed gene expression. Purine/pyrimidine exchanges were generated in the regions of bp −8 to −11 and bp −17 to −20 (Fig. 6a) within a truncated promoter. This mutated 42-bp P*kdp* 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 nonmodified 42-bp promoter. However, exchanges in the region of bp −8 to −11 also resulted in a slight increase in expression even under noninducing conditions. The rather high overall noninduced 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 (from bp −1 to −206) in the same integrative vector background and

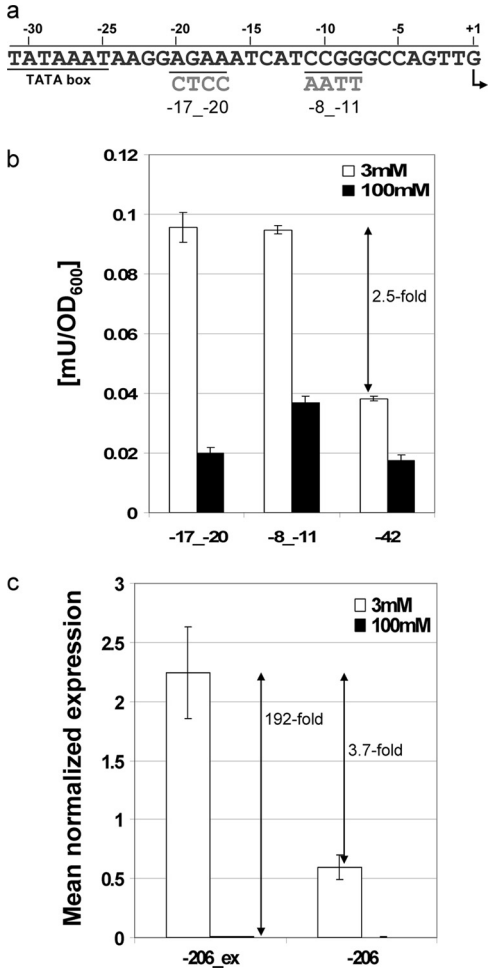

FIG 6 (a) Sequence of P*kdp* between the TATA box and the transcription start site (arrow). Mutations introduced in the regions of bp −17 to −20 (−17_−20) and bp −8 to −11 (−8_−11) are denoted. (b) β-Galactosidase activities of *H. salinarum* R1 Δ*kdpFABC* encoding P*kdp*::*bgaH* fusions with a truncated 42-bp P*kdp* (−42). The bars labeled −17_−20 and −8_−11 indicate the effects of the exchanges shown in panel a. Samples were taken from cultures grown under inducing and noninducing 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 (−206 ex) depicted in panel a. Measurements were performed in triplicate by real-time RT-PCR analysis.

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 P*kdp*, noninduced expression is barely detectable, thus yielding higher overall induction ratios. However, whereas the wild-type P*kdp* achieved an induction ratio of about 50, the concomitant presence of the purine/pyrimidine swaps in the regions of bp −8 to −11 and bp −17 to −20 resulted in an almost-four-times-higher induction ratio of about 190. The swaps did not affect the noninduced expression, thereby demonstrating that the promoter is still tightly regulated.

Taken together, expression levels from P*kdp* 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 on the K^+ concentration in the medium. By use of this key feature of P*kdp*, 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 P*kdp* available for the controlled expression of target genes. The plasmid pKIX enables P*kdp*-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* spp. However, due to its nature of modulation, pKIX is not applicable as a 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 P*kdp*-promoted expression of the β-galactosidase gene *bgaH* were comparable to those of the natural *kdpFABCQ* genes, a finding which already argues for constant P*kdp*-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 noninducing conditions, a result which is in accord with the low basal transcript level observed with an integrative vector (11). This observation implies a less tight repression of P*kdp* under noninducing conditions. However, in our previous studies, we showed 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 P*kdp* resulted in enzyme activities under noninducing 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 significant increases in both expression and the induction ratio were observed in the case of the concomitant presence of *kdpQ* on both plasmid and chromosome. This KdpQ-related enhancing effect may 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 was obtained by the introduction of the purine/pyrimidine swaps in pKIX. The enhanced P*kdp*-derived expression under inducing conditions may 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 bp positions −10 and −11, a finding 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 the region of bp −8 to −11, an "AA" sequence was generated at this position. These inserted "WW" nucleotides might optimize P*kdp* for enhanced expression by enhancing the binding affinity of the transcription initiation components mentioned above. Accordingly, substitutions performed in the region of bp −17 to −20 may 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 have not yet been shown in *H. salinarum*.

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