Transcriptional regulation and physiological importance of the *kdp*-system from the halophilic archaeon *Halobacterium salinarum*

Dissertation
submitted for the degree of

Doctor rerum naturalium
(Dr. rer. nat.)

in the department of Biology / Chemistry
University of Osnabrück

by

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Osnabrück, May 2011

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Preface

This work was financially supported by the Deutsche Forschungsgemeinschaft (grant GR 2698/1-1) and by the Deutsche Forschungsgemeinschaft (SFB431: “Membranproteine: Funktionelle Dynamik und Kopplung an Reaktionsketten“, Teilprojekt P7).

The results are presented in three chapters. Chapter 2 is a submitted manuscript. Chapter 3 reveals pre-studies for a potential publication, whereas the fourth chapter is currently in preparation for publication. These three chapters represent the results of this thesis, which provides new insights into the transcriptional regulation of the *kdpFABCQ* genes and the physiological importance of the *kdp*-system from the halophilic archaeon *Halobacterium salinarum*.

Abbreviations

6'-FAM	6-Carboxy-fluorescein
Ap^R	ampicillin resistance
ADP	adenosine-5´-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
a_W	water activity
BRE	transcription factor B recognition element
cDNA	complementary DNA
DIG	digoxigenin
HTH	helix-turn-helix
INR	initiation region
KdpDN	N-terminal domain of KdpD
MCS	multiple cloning site
Mv^R	Mevinolin / Simvastatin resistance
MOPS	3-(N-morpholino)propanesulfonic acid
OD_x	optical density at a certain wavelength x
ONPG	O-nitrophenyl-β-D-galactopyranoside
ori	origin of replication
PAGE	polyacrylamide-gelelectrophorese
PCR	polymerase chain reaction
P*kdp*	*kdp* promoter
ROX	6-Carboxy-X-rhodamine
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecylsulfate
TBP	TATA box binding protein
TCA	trichloroacetic acid
TFB	transcription factor B
TFE	transcription factor E
Usp	universal stress protein
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
X-Gal	5-bromo-4-chloro-3-indolylβ-D-galactopyranoside
$\Delta\psi_{K+}$	potassium potential
$\Delta\psi_m$	membrane potential

Summary

Obligate halophilic *Halobacterium* species accumulate high concentrations of K^+ in the cytoplasm to maintain an osmotic balance with their hypersaline environments. Therefore, a sufficient uptake of K^+ has to be ensured during growth. The K^+ uptake was supposed to occur merely by low affinity membrane potential-driven K^+ channels and by K^+/H^+-symporters. Nevertheless, the genome *of Halobacterium salinarum* carries genes coding for the high affinity, ATP-dependent K^+ uptake system KdpFABC, which is particularly well characterized in the mesophilic bacterium *Escherichia coli*. The Kdp complex of *E. coli* facilitates growth upon K^+ limitation and expression of the *kdpFABC* operon is highly induced under this condition. This also applies to the *kdp* genes of *H. salinarum*. Whereas high external K^+ concentrations repress transcription of the corresponding halobacterial genes, transcription is initiated at concentrations below 20 mM and increases further until the minimal external K^+ concentrations for K^+ uptake of about 20 µM is reached. These findings give support to the notion that the *kdp* genes are subject to a complex transcriptional regulation. However, sequence analysis of the halobacterial genome revealed the absence of homologues of the two component system KdpD/KdpE, which is involved in transcriptional regulation of the *kdpFABC* operon of *E. coli*. Instead, *H. salinarum* comprises in addition to the structural genes coding for the KdpFABC complex another gene *kdpQ*. KdpQ is involved in transcriptional regulation of the halobacterial *kdp* genes since it stimulates their expression. Due to the assumption that the genes are organized into a putative *kdpFABCQ* operon, KdpQ is supposed to act as a positive co-regulator. Furthermore, *kdpQ* is not essential for a K^+-dependent regulation of the *kdp* genes, which suggests another primary regulatory component to be involved in transcriptional regulation of the halobacterial *kdpFABCQ* genes.

Although K^+ limitation represents a condition for *kdpFABCQ* expression in *H. salinarum*, this feature has apparently a minor physiological significance in hypersaline habitats, since these environments comprise K^+ concentrations well above 100 mM KCl.

Chapter II of this thesis provides insights into the transcriptional regulation of the *kdpFABCQ* genes, whereas Chapter III gives insights into a potential application of the *kdp* promoter as a tool for an inducible expression of halophilic genes in *H. salinarum*. Chapter IV deals with the physiological relevance of the halobacterial Kdp complex.

In order to analyze the regulation of *kdp* gene expression, it was essential to gain information about the transcriptional unit(s) involved. Northern blot analysis revealed the presence of a polycistronic *kdp* transcript confirming the hypothetical *kdpFABCQ* operon. The full-length transcript was well in accord with the determined transcription start site and a putative terminator sequence. Primer extension analyses identified the *kdpFABCQ* transcript as a leaderless transcript with the transcription start site mapped 4bp upstream of the *kdpF* start codon. Real-time RT-PCR analyses in the downstream region of *kdpQ* indicated a putative *kdp* terminator or at least a potential mRNA processing site 85 bp to 110 bp downstream of *kdpQ*. Previous promoter truncation studies revealed the presence of an operator sequence upstream of the basal transcription elements. However, deletions of this putative operator sequence did not lead to a constitutive expression. Further truncation analyses verified the so far only predicted basal transcription elements and excluded also an additional regulatory element upstream of the basal transcription elements. This could account for the rather atypical position of the putative repressor binding site and the low level of repression in the absence of the putative operator. Translational fusions of a halophilic β-galactosidase to the *kdp* promoter resembled an expression pattern similar to the natural *kdp* gene expression, which excluded a second operator binding site in the *kdp* coding region. Scanning mutagenesis analyses in the region between the TATA box and the transcription start site furthermore provided no obvious support for a putative additional regulatory element. However, transfer of the halobacterial *kdpFABCQ* operon to another halophilic archaeon, *Haloferax volcanii* (which naturally does not encode the *kdp* genes) revealed expression patterns similar to those of *H. salinarum*, in which the putative operator sequence is deleted. Therefore the *kdpFABCQ* expression is assumed to be at least partially conserved among halophilic archaea, leading to speculations of multiple basal transcription factors to be involved. Since the *kdp* genes in *H. volcanii* improve cell growth upon K^+ limiting conditions indicating a functional Kdp complex in this organism, measurements of the residual K^+ concentration confirmed the affinity limit of the Kdp complex of 20 µm K^+ as determined in *H. salinarum*.

An inducible expression system for e.g. conditional lethal mutants or synthesis of putative toxic proteins is so far missing for *Halobacterium salinarum*. The halobacterial *kdpFABCQ* operon is

highly upregulated upon K^+-limiting conditions and is repressed by high extracellular K^+ concentrations. Since the dependency on external K^+ influences *kdp* gene expression, the use of the *kdp* promoter would enable a system in which the halobacterial gene expression could be controlled by simple manipulation of growth conditions. Therefore, a replicating vector (pKIX) containing the promoter of the *kdpFABCQ* operon was constructed, which allows a controlled gene expression in *H. salinarum*. pKIX is functional and enables a promoted K^+-dependent expression from the *kdp* promoter with rather high induction ratios of 50-fold. Furthermore, expression levels could be improved by plasmid- and additional chromosomally encoded *kdpQ*, since in these cases an increase in the expression and induction ratio could be observed. Furthermore, mutations generated in the *kdp* promoter between the TATA box and the transcription start site also had a positive influence on the pKIX-mediated expression rate. Since transcript levels from pKIX were found to be independent of differential target genes, the general application of pKIX as an inducible expression system is strongly supported.

K^+ is a common ion in hypersaline environments and available in concentrations not limiting for halobacterial cell growth. However, hypersaline habitats are usually exposed to high temperatures and solar radiation resulting in concentration of brines accompanied by halite formation. To gain information about the physiological relevance of the *kdp* system, studies were performed to test whether the *kdp* operon has an influence on desiccation stress. Halite crystals grown under non-inducing K^+ concentrations with embedded strains of *H. salinarum* and *H. salinarum* deleted in the *kdpFABCQ* genes revealed a significantly reduced survival rate of the deletion strain. Additionally, a *kdpFABCQ*-inducing desiccation stress could already be determined on agar plates under non-limiting K^+ concentrations. Furthermore, the cell morphology of *H. salinarum* entrapped in halite crystals resembled those of *H. salinarum* grown upon K^+ limiting conditions. Therefore, the *kdp*-system promotes survival of *H. salinarum* under desiccation stress.

Chapter I

General Introduction

General Introduction

Hypersaline ecosystems

Hypersaline environments like salt flats, solar salterns and natural salt lakes (Javor, 1989) prevalently originated from the evaporation of seawater. These environments are designated as *thalassohaline* (greek *thálassa* = 'sea') since NaCl is the dominating salt together with an overall proportional composition of ions similar to seawater and a neutral to slightly alkaline pH (Larsen, 1980). Evaporetic pools can develop naturally from ocean margins in arid areas or are artificially induced for salt production. As water evaporates in these pools, a sequential precipitation of minerals occurs starting with calcium carbonate ($CaCO_3$, calcite), calcium sulfate ($CaSO_4 \cdot 2H_2O$, gypsum), whereafter a hypersaline sodium chloride brine remains. Afterwards, sodium chloride precipitates as halite (NaCl) and the remaining liquid is primarily composed of magnesium chloride which represents hostile bittern brines (Brock, 1979; Grant, 2004).
However, hypersaline waters classified as *athalassohaline* are not dominated by NaCl as the main salt and resemble a different proportion of seawater ions. These hypersaline brines develop influenced by the geology of the area, for example by extensive leaching of the alkaline earth metals magnesium and calcium (Oren, 1994). For instance, the ion composition of the Dead Sea is highly influenced by leaching of magnesium from geologically ancient salt deposits (Eugster & Hardie, 1978).
The divalent cations calcium and magnesium are further involved in the determination of the brines' pH. In the case of the *athalassohaline* Dead Sea the precipitation of sepiolite ($MgSiO_8 \cdot nH_2O$) besides magnesite ($MgCO_3$) and dolomite ($CaMg(CO_3)_2$) generates H^+ which causes slightly acidic conditions.
Generally, the pH of seawater is determined by the equilibrium between carbonate (CO_3^{2-}), hydrogencarbonate (HCO_3^-) and carbon dioxide (CO_2). The precipitation of calcite in *thalassohaline* lakes removes the alkaline carbonate. However, the relatively high concentrations of calcium keep the brines' neutrality since the amount of calcium exceeds that of carbonate (Grant, 2004).
Instead, high salinity soda lakes with pH values up to 12 evolve as a result of scarcity of magnesium and calcium in the water chemistry causing an increase of the alkaline carbonate (Jones, 1998).
Although the hypersaline waters are diversified in the ion composition and pH, life from nearly all three domains can be found throughout the different specified environments.
Although general survival of higher eukaryotic organisms is avoided by high salinities, the eukaryotic brine shrimp *Artemia* is as an exception able to thrive in extremely hypersaline brines up to NaCl saturation albeit not in extremely alkaline types (Jorgensen & Amat, 2008; Grant, 2004). Furthermore, lower eukaryotes are also relatively scarce in hypersaline environments. However, the phototrophic, halotolerant alga of the genus *Dunaliella* is widely distributed in *thalassohaline* as well as in *athalassohaline* environments with NaCl concentrations ranging from 0.05 M to saturation (Chen & Jiang, 2009, Oren 1994).
Despite the existence of few eukaryotic organisms, hypersaline environments are predominantly inhabited by prokaryotes. *Salinibacter ruber* as an example of hypersaline bacteria can be found in saltern crystallizer ponds with NaCl concentrations from at least 2.5 M up to saturation (Antón *et al.*, 2002). However, the climax population of hypersaline lakes at the point of halite precipitation belongs to the group of haloarchaea.
The extremely halophilic archaea (also called haloarchaea) belong to the order Halobacteriales, which contains one family, the *Halobacteriaceae* (Grant *et al.*, 2001), with 27 accredited genera and 96 classified species (Oren *et al.*, 2009). Among these organisms five genera can be found which are exclusively alkaliphilic (*Natronobacterium, Natronococcus, Natronomonas, Natronolimnobius* and *Halalkalicoccus*). Furthermore, species of the genera *Haloarcula*, *Halobaculum*, *Haloferax*, *Halobiforma, Natronorubrum*, *Natrialba* as well as *Halorubrum* comprise both alkaliphilic and neutrophilic species (Gareeb & Setati, 2009). However, the majority of haloarchaeal organisms thrive in neutral environments represented e.g. by most species of *Halobaculum* and *Halorubrum* as well as *Halobacterium*.
In contrast to hypersaline lakes and solar salterns, knowledge of further high salt environments like e.g. hypersaline soils and salt marshes is marginal (Grant *et al.*, 2004).
Most halophilic archaea are pigmented red due to a high content of C-50 carotenoid pigments (α-bacterioruberin and derivatives) in their membrane, in some cases accompanied by the purple retinal pigment bacteriorhodopsin (Kushawa & Kates, 1979; Oren, 2002). Furthermore, *Dunaliella*

produces high amounts of β-carotene (Ben-Amotz & Avron, 1983) and also *Salinibacter ruber* is pigmented due to C-40 carotenoids (Lutnaes *et al.*, 2002). Dense blooms of these halophilic organisms colour neutral and alkaline saturated hypersaline lakes red (Grant *et al.*, 2004) and further encourage evaporation by trapping solar radiation. As NaCl precipitates halophilic organisms become trapped inside halite's fluid inclusions, which can constitute 2-6% (w/w) of freshly harvested solar salt (Lefond, 1969). The organisms' ability to be captured and survive in these brine inclusions could represent an important strategy for survival (Norton & Grant, 1988). Halophilic archaea trapped in halite are motile for several weeks and can be recovered after years or even millions of years (Oren, 1994; McGenity *et al.*, 2000).

Adaptation to saline environments

The thermodynamical availability of water is a contributing factor for life. The overall high concentration of solutes in the hypersaline environments mainly determined by NaCl (2.5 M to saturation) results in a low water availability with a water activity (a_W) of 0.75 (Grant *et al.*, 2004). Since biological membranes are permeable to water, cells have to maintain their cytoplasm at least isoosmotic with its environment to prevent a loss of water. Dehydration of cells would have a severe impact on growth, possibly due to molecular crowding attended by reduced diffusion rates of proteins and metabolites (Kunte *et al.*, 2002). Most halophiles as well as almost all organisms maintain the cytoplasmic osmolality higher than that of the surrounding medium, resulting in the diffusion of water into the cell and, as a consequence, in generation of turgor. However, halophilic archaea within the family of *Halobacteriaceae* lack a significant turgor pressure since they only maintain isoosmotic equilibriums with their surroundings (Walsby, 1971).
Two principle mechanisms have evolved on earth to cope with the osmotic stress in environments with high salinity: the *compatible solute* and the *salt-in* strategy. The *compatible solute* strategy is commonly used by the most halophilic and halotolerant microorganisms. To provide the essential osmotic balance, small organic molecules are either accumulated from the medium or are even synthesized by the cells. The types of *compatible solutes* used for osmoprotection for example include polyols (e.g. glycerol, arabitol), sugars and sugar derivatives (e.g. sucrose, trehalose) as well as amino acids and their derivatives (e.g. glycine-betaine) (Madigan & Oren, 1999; Roberts, 2005). *Compatible solutes* are typically low-molecular-weight compounds which are soluble at high concentrations, either uncharged or zwitterionic at neutral pH and do not interfere with any enzyme function (Brown, 1990). The *compatible solutes* strategy is energetically unfavorable for organisms thriving at salt concentrations above 1.5 M NaCl, since the synthesis of organic compounds is highly energy consuming: with an average of 30 to 109 mole ATP for the synthesis of 1 mole solute to counterbalance 1 mole NaCl (Oren, 1999; Oren, 2008).
As an alternative, halophilic archaea of the order Halobacteriales and the phylogenetically unrelated anaerobic halophilic bacteria of the order Haloanaerobiales balance their cytoplasm via the *salt-in* strategy (Oren, 1999). These organisms accumulate molar intracellular ion concentrations similar to their surrounding. In contrast to the medium with NaCl as main salt, KCl is the preferred ion to be intracellularly accumulated (Dennis & Shimmin, 1997). In contrast to K^+ ions, Na^+ has a higher surface charge density due to its smaller ionic radius and is therefore more heavily hydrated. Therefore, Na^+ ions bind a greater amount of water molecules than K^+ ions. The preference of K^+ towards Na^+ within cells is probably due to this feature, since there is a strong competition of ions and protein surfaces for the available water molecules (Chaplin, 2006).
As a result of molar intracellular KCl concentrations, cells using the *salt-in* strategy have to adapt all enzymes and structural components to ensure the proper functioning of the intracellular machinery (Oren, 1999; Grant, 2004). Haloarchaeal proteins exhibit a unique molecular adaptation due to small amounts of hydrophobic and a large excess of acidic amino acids (glutamate and aspartate) especially on the protein surface, combined with a low content of basic amino acids (lysine and arginine) (Lanyi, 1947; Mevarech *et al.*, 2000; Oren 2002). These features provide the proteins' solubility with respect to a dehydrating effect by the high ion concentration of the surrounding (Dennis & Shimmin, 1997). Furthermore, it has been suggested that the intracellular high levels of cations are important for an interplay with the negatively charged proteins (Mevarech *et al.*, 2000). Microorganisms relying on the *salt-in* strategy are therefore dependent on the continuous presence of high salt concentrations in their environment, since most enzymes and other proteins denature when they are exposed to low ionic strength (1–2 M) (Kushner, 1978). This is most likely the reason why all known organisms adapting to high salinities by using the *salt-in* strategy are obligate halophilic (Lanyi, 1974; Oren 1999). Although the *salt-in* strategy requires only 0.5 to 0.67 mole ATP to counterbalance 1 mole NaCl with 1 mole KCl, organisms using this strategy are, as already mentioned, not widely

distributed within the different phylogenetic and physiological groups of halophiles (Oren, 1999; Oren, 2008).

Halobacterium salinarum and ion metabolism

The obligate halophilic archaeon *H. salinarum* of the order Halobacteriales represents besides *Haloferax volcanii* the haloarchaeal model organism. *H. salinarum* exhibits an optimal growth in NaCl concentrations of 3.9 M ($NaCl_{range}$ 2.2-5.2 M) and a temperature optimum of 50°C (T_{range} 10-50°C) (Zeng *et al.*, 2006, Coker *et al.*, 2007; Robinson *et al.*, 2005). This organism is ubiquitous in *thalassohaline* environments like neutral salt lakes and solar salterns and is a common contaminant on heavily salted food e.g. fish, from which it was also firstly isolated (salted codfish showing a red discoloration (Harrison & Kennedy, 1922)). *H. salinarum* is rod-shaped but displays several pleomorphic forms (bent and swollen rods, clubs and spheres) especially in NaCl-deficient media (Mesbah & Wiegel, 2005). Although there are carbohydrate-utilizing species within the haloarchaea, *H. salinarum* and some others are not capable to metabolize sugars (Rawal *et al.*, 1988). Instead, *H. salinarum* is growing on the degradation products of other organisms. Amino acids are utilized during aerobic growth as energy source and are ultimately catabolized via the citric acid cycle (Ng *et al.*, 2000). However, oxygen is a limiting component in hypersaline environments since it is poorly soluble in concentrated brines but under anaerobic conditions *H. salinarum* is able to grow fermentatively on arginine via the deiminase pathway or by anaerobic respiration on fumarat, trimethylamine N-oxide (TMAO) or dimethylsulfoxide (DMSO) (Oren, 2006, Ruepp & Soppa, 1996). Furthermore, under low oxygen tension *H. salinarum* is able to generate a proton motive force through the light-dependent bacteriorhodopsin cycle involving retinal, which can be further used to drive ATP synthesis through action of the proton-translocating A_1A_O ATPase (Haupts *et al.*, 1999; Pfeiffer *et al.*, 2008).

The genome sequences of two isolates of *Halobacterium salinarum* are available – *H. salinarum* NRC-1 and *H. salinarum* R1 (Ng *et al.*, 2000; Pfeiffer *et al.*, 2008). Both genome sequences are so similar in sequence that they most likely originate from the same cultivation event of a natural isolate (Pfeiffer *et al.*, 2008).

However, *H. salinarum* uses, as discussed above, the *salt-in* strategy to maintain an isoosmotic equilibrium with its surrounding (Oren, 1999). Therefore, an efficient uptake of K^+ combined with an extrusion of Na^+ has to be ensured. The genome of *H. salinarum* encodes a variety of ion transport proteins (Fig. 1). Four copies of NhaC (NhaC1-NhaC4), a Na^+/H^+ antiporter which uses the proton electrochemical gradient as driving force for the extrusion of Na^+ in a stoichiometry $2H^+/Na^+$, keep the intracellular Na^+ concentration rather low (0.8-1.63 M Na^+, Christian & Waltho, 1962; Ginzburg *et al.*, 1970; Matheson *et al.*, 1976) (Lanyi & Silverman, 1979, Pfeiffer *et al.*, 2008). The passive permeability of haloarchaeal membranes for Na^+ is very low (Vosseberg, 1999) but rather high for K^+ (Lanyi & Hilliker, 1976). K^+ can thus enter the cell via a uniport system which constitutes most likely the primary K^+ uptake mediated by the existing membrane potential ($\Delta\psi_m$) (Lanyi *et al.*, 1979). According to the Nernst equation, passive K^+ uptake is feasible till the K^+ potential ($\Delta\psi_{K+}$) is less negative or equal to the

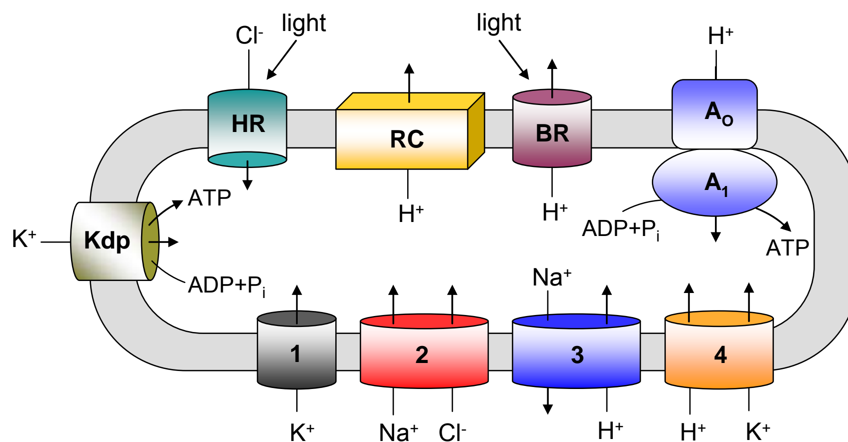

Fig. 1. Schematic diagram of the monovalent ion transport systems in *H. salinarum*.
A_OA_1: ATP-generating proton-driven A_OA_1 ATPase; BR: light-driven proton pump bacteriorhodopsin; RC: electron-transport driven proton-translocating respiratory chain; HR: light-driven Cl^- pump halorhodopsin; Kdp: ATP-driven K^+ uptake system KdpFABC; 1: inward-directed K^+ uniport via channels PchA1/PchA2; 2: Cl^- uptake via Na^+ symport; 3: Na^+ export coupled to H^+ import system NhaC1-4; 4: inward-directed symport of H^+ and K^+ via the TrkAH system.

$\Delta\psi_m$. In the case of a more negative $\Delta\psi_{K+}$ than the present $\Delta\psi_m$, K^+ transport has to be energized either by co-transport with other ions or by ATP hydrolysis.

The proteome (according to the genome sequence) of *H. salinarum* exhibits two putative K^+-channels (PchA1 and PchA2) and four homologues of the bacterial TrkAH system (TrkH1-TrkH4) which represent H^+/K^+ symporters with moderate to rather low affinities for K^+. In the Trk system of *E. coli* ion transport is mediated cooperatively via ATP (Bakker, 1993; Stumpe *et al.*, 1996). However, ATP is not used as the energy source of the transporter but, rather, as a regulator of its activity (Stewart *et al.*, 1985). The putative PchA cannels and the TrkAH system in *H. salinarum* most likely support the cells essential K^+ uptake till the opposing membrane potential is balanced ($\Delta\psi_m=\Delta\psi_{K+}$). To warrant a further uptake of K^+ the transport has to be energized independently of the membrane potential. Although an ATP-driven transport within the order Halobacteriales was excluded for a long period of time (Oren, 1999), the genome sequence unraveled the existence of homologues for the bacterial KdpFABC complex (Pfeiffer *et al.*, 2008). The K^+ uptake system KdpFABC constitutes a high-affinity P-type ATPase mediating K^+ transport via the hydrolysis of ATP (Bramkamp *et al.*, 2007).

To balance the high intracellular monovalent cation concentration, Cl^- serving as counter ion is accumulated in the cytoplasm of *H. salinarum*. Cl^- uptake is reported to occur via two energy-dependent chloride pumps. Halorhodopsin, a further retinal-protein, enables Cl^- uptake by light (Schobert & Lanyi, 1982) whereas another light-independent transport system, probably driven by symport with Na^+ (Duschl & Wagner, 1986), additionally ensures the Cl^- uptake.

Furthermore, knowledge about the intracellular concentrations of divalent ions (e.g. Ca^{2+} and Mg^{2+}) and their mechanisms of uptake and/or extrusion is marginal (Oren, 2002a). Anyway, an outward calcium transport system most likely acting as a Na^+/Ca^{2+} antiporter has been identified in *H. salinarum* (Belliveau & Lanyi, 1978).

The KdpFABC complex of E. coli *and* H. salinarum

H. salinarum is the only halophilic archaea to date which encodes genes for the Kdp complex (Pfeiffer *et al.*, 2008a). The KdpFABC complex represents a P-type ATPase responsible for a high-affinity inward-directed K^+ transport energized by the hydrolysis of ATP. Although the K^+ uptake system Kdp can be found in several other non-halophilic archaea, knowledge of this P-type ATPase is restricted to bacteria.

Like all members of the P-type ATPase family, the KdpFABC complex is also characterized by a phosphorylated intermediate during the catalytic cycle and inhibition by *ortho*-vanadate (Siebers & Altendorf, 1988). However, within the group of P-type ATPases the Kdp complex exhibits some extraordinary features. In many eukaryotic organisms, P-type ATPases comprise only one subunit. In contrast, the Kdp complex is composed of four subunits. However, multiple polypeptides forming the transport system can occasionally be found in other bacterial P-type ATPases. A genuine unique feature of the KdpFABC complex among P-type ATPases is that the catalytic activity and the substrate transport (here: K^+) are located on two different subunits (Bramkamp *et al.*, 2007).

Within the Kdp complex of *E. coli*, KdpA is responsible for K^+ binding and transport (Buurman *et al.*, 1995). However, to facilitate the K^+ transport, energy is provided by KdpB via ATP hydrolysis at the nucleotide-binding domain (Haupt *et al.*, 2006), which is postulated to be supported by the functionally essential subunit KdpC acting as a catalytical chaperone (Ahnert *et al.*, 2006; Greie & Altendorf, 2007). Although deletion mutants of *kdpF* showed no decrease in transport activity *in vivo*, this small (3kDa) hydrophobic subunit is important for a functional Kdp complex regarding ATPase activity *in vitro* (Gaßel *et al.*, 1999). Therefore, KdpF is suggested to have a lipid-like stabilizing function on the Kdp complex (Greie & Altendorf, 2007).

In *E. coli*, the Kdp system is composed of the KdpFABC complex and the dedicated regulatory two component system KdpD and KdpE (Fig. 2) (Jung & Altendorf, 2002). The structural genes *kdpFABC* are organized into a polycistronic operon, which together with the corresponding *kdpDE* operon forms the *kdp* regulon (Völkner *et al.*, 1993). The Kdp complex of *E. coli* facilitates growth upon K^+ limitation, and expression of the *kdpFABC* operon is highly induced under this condition. Furthermore, expression of the *E. coli kdp* genes is induced by high osmolality in the medium, however to a minor extend (Hamann *et al.*, 2008). Whereas KdpD acts as the membrane-bound sensor kinase sensing the still controversially discussed stimulus, KdpE constitutes the cytoplasmic response regulator. Upon stimulus perception, KdpD undergoes ATP-dependent autophosphorylation at a conserved histidine residue (His_{673}) and the phosphoryl group is subsequently transferred to a conserved aspartate (Asp_{52}) in KdpE (Völkner *et al.*, 1993). Phosphorylated KdpE dimerizes and finally binds as a classical helix-turn-helix type transcriptional activator to the promoter of the *kdpFABC* operon (Nakashima *et al.*, 1993). However, in the absence of the stimulus, non-phosphorylated KdpD is able to dephosphorylate KdpE which, as a result,

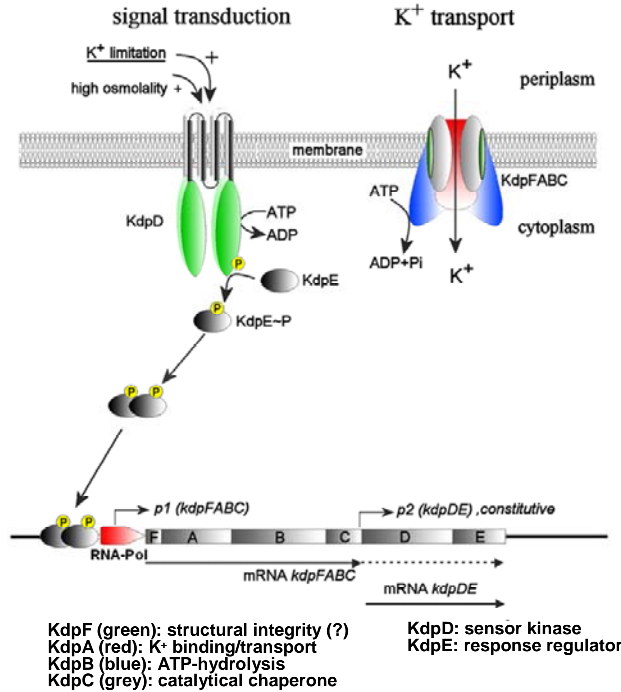

Fig. 2. Overview of the Kdp system in *E. coli*.
Left: Interplay of the sensor kinase/response regulator pair KdpD/KdpE in transcriptional regulation of the *kdpFABCDE* regulon (lower panel). Right: the membrane bound K^+ translocating KdpFABC complex.

terminates *kdpFABC* expression (Jung *et al.*, 1997).
The *kdpFABC* genes of *H. salinarum* are apparently organized in one single operon like in *E. coli* (Fig. 3) (Strahl & Greie, 2008). Sequence analysis of the halobacterial genome revealed the absence of homologues for the sensor kinase/response regulator pair KdpD/KdpE. However, the *kdpFABC* genes of *H. salinarum* seem to be co-expressed with an additional gene *kdpQ*. Structural homology modelling revealed that KdpQ resembles a tandem universal stress protein (Usp) with no further domains or extensions, and at least the C-terminal motive shows high homology to Usp domains of the ATP-binding type (Strahl & Greie, 2008). Deletion studies of *kdpQ* revealed that KdpQ is not essential for a K^+-dependent gene regulation although it stimulates *kdpFABCQ* gene expression. Since *kdpQ* is suggested to be co-expressed together with the structural genes, KdpQ most likely acts as a positive co-regulator (Strahl & Greie, 2008). Since *E. coli* KdpD also contains a Usp domain of a putative ATP binding type (Siegele, 2005; Zimmann *et al.*, 2007), a functional homology of these domains in *E. coli* KdpD and *H. salinarum* KdpQ regarding their ATP-binding properties has already been discussed (Strahl & Greie, 2008).
Like in *E. coli*, the KdpFABC complex of *H. salinarum* also promotes growth under K^+-limiting conditions. Although the regulatory components of *H. salinarum* and *E. coli* are obviously different, the *kdp* gene expression patterns upon K^+ limitation are virtually identical in both organisms (Strahl & Greie, 2008). Whereas high external K^+ concentrations repress transcription of the halobacterial *kdpFABCQ* genes, transcription is initiated at concentrations below 20 mM and increases further until a minimal external K^+ concentration of about 20µM is reached (Strahl &

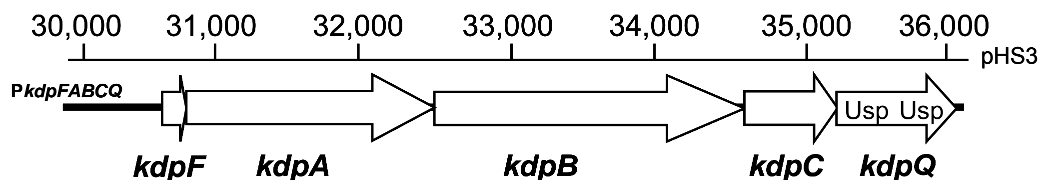

Fig. 3. Organization and length of the putative *kdpFABCQ* operon in *H. salinarum*.
The numbers indicate the localization of the genes on the extrachromosomal pHS3 of *H. salinarum* R1 (Pfeiffer *et al.*, 2008).

Greie, 2008). Deletion studies of the putative *kdpFABCQ* operon revealed that the Kdp complex of *H. salinarum* is essential for growth below concentrations of 60 µM external K^+. Concomitant to external K^+-limiting conditions, the internal K^+ concentration is decreased by approximately 30% to a minimal value of 2.8 M (Strahl & Greie, 2008).

Archaeal transcription

The basal transcription machinery of archaea is highly similar to that of eukaryotes (Zillig *et al.*, 1978; Langner *et al.*, 1995). Commonly in all three domains of life, general transcription initiation factors are needed for recruitment of the RNA polymerase to the DNA promoter, which together form the basal transcription machinery or preinintiation complex. In bacteria a dissociable sigma factor (δ) has to join the core RNA polymerase (consisting of four subunits, α2ββ′) to form the RNA polymerase holoenzyme (α2ββ′δ) (Buck *et al.*, 2000). Once the holoenzyme is assembled, this preinitiation complex it is able to recognize the promoter DNA at positions around -35 and -10 bp upstream the transcription start site (Haugen *et al.*, 2008). In contrast, eukaryotes and archaea possess a larger quantity of general transcription initiation factors also known as basal transcription factors. In the case of the eukaryotic RNA polymerase II, six general transcription factors (TFIIA, TFIIB, TFIID [composed of TATA box binding protein, TBP, and numerousness TBP associated factors], TFIIE, TFIIF and TFIIH) together with a mediator complex and the RNA polymerase II form the preinitiation complex (Hampsey, 1998; Hahn, 2004). Although similar to the eukaryotic basal transcription machinery, the archaeal version is much simpler. The transcription preinitiation complex of archaeal organisms contains the RNA polymerase together with TBP, TFB and TFE, which are orthologs of the eukaryal TBP, TFIIB and TFIIE, respectively (Bell & Jackson, 1998). Furthermore, the archaeal RNA polymerase is a protein complex with at least 10 subunits (dependent on the species). Structural analysis combined with sequence alignments revealed high homology to the RNA polymerase II of eukaryotic organisms, which consists of up to 15 subunits (Hirata *et al.*, 2008; Bell & Jackson, 1998; Bell & Jackson, 2001).
RNA polymerases are incapable of sequence-specific promoter recognition and have to be recruited via the basal transcription factors. In archaea, the first protein to bind DNA is TBP, which recognizes an AT-rich TATA box sequence within the promoter region located -25 to -30bp upstream the transcription start site (Fig. 4) (Hausner & Thomm, 1995; Gohl *et al.*, 1995; Brenneis *et al.*, 2007). TBP causes a sharp DNA

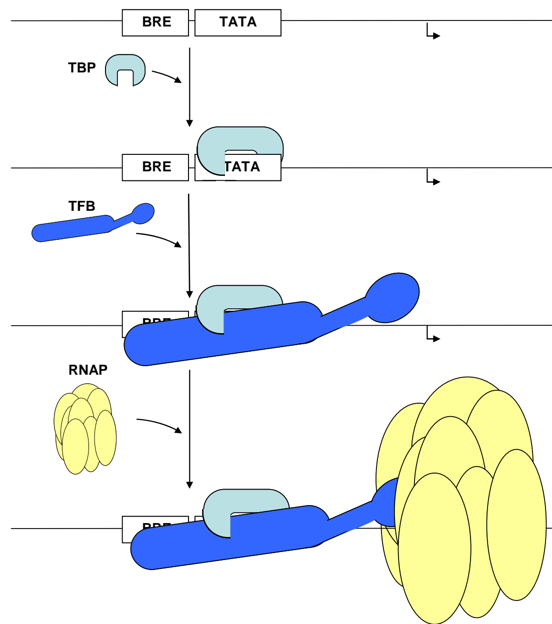

Fig. 4. Schematic overview of transcription initiation in archaea. RNAP represents the multisubunit RNA polymerase. The transcription start site is depicted by an arrow. Modified according to Bell & Jackson (1998) and Thomm (2007).

bend (Kosa *et al.*, 1997), which might enable recruitment of the transcription factor TFB at the purine rich transcription factor B recognition element (BRE) upstream of the TATA box (Thomm, 2007, Jun *et al.*, 2011). However, TFB interacts not solely with promoter DNA in the region around the TATA-box but also in the near vicinity of the transcription start site (Renfrow *et al.*, 2004). Binding of TFB to the promoter region at BRE is reported to influence the level of basal transcription and further defines the transcriptional orientation (Littlefield *et al.*, 1999). This ternary complex (DNA, TBP and TFB) recruits the RNA polymerase, which interacts with TFB and the DNA in the downstream region of the TATA-box up to position +18 (Thomm, 2007), thereby initiating transcription.
Whereas the archaeal basal transcription factors TBP and TFB are essential for transcription initiation, the third less-well characterized general transcription factor TFE is not absolutely required at least *in vitro*. However, TFE stimulates transcription in cases where the TBP or TFB function is not optimal, at least in part by stabilizing the open complex, in which the DNA strands surrounding the transcription start site are separated (Hanzelka *et al.*, 2001; Bell *et al.*, 2001). To fulfill this function, archaeal TFE is capable to interact directly with TBP and RNAP and can bind to single-stranded DNA via a winged helix-turn-helix motif (Bell *et al.*, 2001; Grünberg *et al.*, 2007; Meinhart *et al.*, 2003).

Transcriptional regulation in archaea

To control gene expression under dedicated conditions, organisms encode large numbers of different DNA-binding proteins to positively or negatively regulate transcription initiation. DNA-binding transcription factors can be classified into diverse families according to their amino acid sequence. Comparative analysis of numerous archaeal transcription factors revealed the presence of transcriptional regulators clearly associated with the bacterial type (Pérez-Rueda & Janga, 2010; Aravind & Koonin, 1999). Like in bacteria, helix-turn-helix (HTH) domains are the most abundant domains in archaeal DNA-binding proteins. Even though HTH motifs are also present in eukaryotic DNA-binding domains like for instance in homeodomains, Paired and POU domains, the archaeal HTH domains are much more similar in sequence to bacterial HTH domains (Aravind & Koonin, 1999). Accordingly, homologues of almost 50% of archaeal transcription regulators can also be found in bacteria, whereas only 1.6% are common between archaea and eukaryotes. However, another minor fraction of transcription factors can be found in all three domains but nevertheless a large quantity (almost 45%) is unique to archaea. The archaeal transcription factors are grouped in diverse families mainly represented by members of ArsR, Lrp/AsnC, HTH_3 and TrmB (Pérez-Rueda & Janga, 2010).

The combination of a eukaryal type transcription machinery and transcriptional regulators mainly of the bacterial type raises the question of how these components interact or interfere in archaeal cells (Aravind & Koonin, 1999; Kyrpides & Ouzounis, 1999). The study of transcription regulation in archaea is at its infancy since only a few archaeal transcriptional regulators have been examined in detail so far.

Within the poorly identified archaeal transcriptional regulators, research mainly focused on members of the Lrp (leucine-responsive regulatory protein) family, which is, therefore, the best studied family of archaeal regulators. Lrp-like proteins are a well-known regulator family in bacteria and have important functions in *E. coli*, e.g. in amino acid metabolism (Calvo & Matthews, 1994). The family of Lrp-like proteins is very diverse, since it constitutes either global or gene-specific regulatory proteins. Furthermore, this regulator can act as an activator or a repressor via an effector molecule (mainly leucine) or independently of an effector (Calvo & Matthews, 1994; Newman & Lin, 1995; Brinkman *et al.*, 2003). As examples of the Lrp-family, *Pyrococcus* LrpA and Lrs14 of *Sulfolobus solfataricus* inhibit transcription of their own genes. Binding of *Pyrococcus* LrpA is centered at the transcription start site, thereby not interfering with TBP and TFB but preventing RNA polymerase recruitment. Instead, Lrs14 of *S. solfataricus* binds to sequences overlapping with the basal transcription factors and thereby blocking overall transcription initiation (Dahlke & Thomm, 2002; Bell & Jackson, 2000). An example of an Lrp-like archaeal transcription factor, which most likely constitutes an activator dependent on ligand binding, is LysM of *S. solfataricus*. LysM regulates biosynthesis of lysine and binds to the *lysW* promoter upstream of BRE. However, in the absence of lysine, DNA-binding is stronger, thereby most likely triggering expression of lysine biosynthesis genes (Brinkman *et al.*, 2002).

MDR1 of *Archaeoglobus fulgidus* is a homologue of the bacterial metal-dependent repressor DtxR (DtxR family). This repressor binds to its own promoter via three operator sequences located between -18 and +67 assisted by the presence of Fe^{2+}, Mn^{2+} and Ni^{2+}. MDR1 does not interfere with TBP and TFB binding but prevents RNA polymerase recruitment (Bell *et al.*, 1999).

TrmB of *Pyrococcus furiosus* is a global regulator of the TrmB-family interfering at least with two promoters each responsible for transcription of an ABC transporter. The first transporter, the TM system, mediates specific uptake of trehalose and maltose, whereas the second transporter, the MD system, is responsible for maltodextrin uptake. TrmB is regulated via differential ligand binding. In case of the TM system, binding of maltose and trehalose leads to dissociation of the repressor which binds to an operator overlapping with the BRE-TATA box in front of the first gene. However, in the MD system TrmB binds to an altered operator overlapping with the transcription start site of the first gene of the operon. Here, the ligands used for TM system induction have no effect but maltose and trehalose are the inducers in this system. However, in the presence of glucose TrmB has an increased affinity for the operator concerning the TM and MD system, thereby concomitantly increasing the repression of these operons. Via this mechanism only one sugar carbon source can be utilized, which is most likely mediated via different specific conformational changes as a result of binding to different ligands (Lee *et al.*, 2005; Lee *et al.*, 2007).

A novel type of repressor family is NrpR of *Methanococcus maripaludis*. Although this protein constitutes a HTH domain, homologues can be exclusively found within the phylum euryarchaeota. NrpR controls expression from the *nif* operon responsible for nitrogen fixation by binding to two operator sequences downstream of the transcription start site. Transcription of *nif* genes is under the control of the effector 2-oxoglutarate. This molecule is an intracellular indicator for nitrogen deficiency and lowers the affinity of NrpR

to its operator sequences (Lie & Leigh, 2003; Lie et *al.*, 2005).
Another activator protein, which is involved in synthesis of gas vesicles in *H. salinarum,* is GvpE. This protein activates two oppositely oriented promoters by binding to a conserved sequence upstream of BRE (Bauer *et al.*, 2008). GvpE interacts with another regulatory protein GvpD. This protein diminishes GvpE activity and is therefore a transcriptional repressor in this system (Hofacker *et al.*, 2004; Zimmermann & Pfeifer, 2003; Scheuch & Pfeifer, 2007). A special feature of GvpE is that this protein contains a leucine zipper motif (bZIP), which is similar to the eukaryal activator GCN4 (Krüger *et al.*, 1998).
Furthermore, additional transcriptional regulators have been identified which are not mentioned here.
Besides diverse transcriptional regulators of different families, several archaea encode multiple homologues of TBP and/or TFB. Especially members of the family *Halobacteriaceae* feature numerousness basal transcription factors with a very complex set in *H. salinarum* (*H. salinarum* R1: 4xTBP and 8xTFB; *H. salinarum* NRC-1: 6xTBP and 7xTFB). Therefore, it has been suggested that the multiple TBP and TFB proteins can form alternative TBP-TFB-RNA polymerase complexes which initiate transcription at different promoters or under different growth conditions. This mechanism in gene regulation is similar to the multiple sigma factors involved in bacterial gene regulation (Baliga *et al.*, 2000).
This view is supported by the fact that *H. salinarum* NRC-1 strains with gene knockouts of *tbpA*, *tbpC*, *tbpD* or *tbpF* and *tfbA*, *tfbC* or *tfbG* could be obtained and further showed differential gene expression patterns. In contrast, strains deleted in the remaining *tbp* and *tfb* genes could not be obtained, leading to the assumption that these proteins are essential for growth at least under standard laboratory conditions (Facciotti *et al.*, 2007; Coker & DasSarma, 2007).

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Chapter II

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

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

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Running title: Transcriptional regulation of the halobacterial *kdpFABCQ* genes

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This chapter is published as:

Kixmüller D, Strahl H, Wende A, Greie J-C (2011) Archaeal transcriptional regulation of the prokaryotic KdpFABC complex mediating K^+ uptake in *H. salinarum*. *Extremophiles* 15:643–652
DOI: 10.1007/s00792-011-0395-y

Due to the publication procedure by a peer-reviewed journal is the published version a slight modified form of the thesis submitted for examination.

Acknowledgements

Ursula Krehe is acknowledged for technical assistance. Further, we thank Ertan Özyamak for construction of plasmid pDSK1. This work was supported by the Deutsche Forschungsgemeinschaft (grant GR 2698/1-1).

Note:

Initial analyses regarding the determination of the *kdpFABCQ* transcription start site and the putative *kdpFABCQ* terminator were performed during my diploma thesis. Information regarding the presence of an operator involved in *kdpFABCQ* regulation is also reported in this work (D. Kixmüller (2006) “Untersuchungen zur Promotorstruktur des *kdpFABCcat3*-Operons von *Halobacterium salinarum*”, diploma thesis, University of Osnabrück). On the basis of these results, H. Strahl was able to construct the plasmid encoding the *kdp* promoter with an operator deletion fused to *bgaH* and the *kdpFABCQ* genes during his PhD work.
Studies concerning a potential function of two-component systems to be involved in transcriptional regulation of the *kdpFABCQ* operon as well as information about a global transcription response were derived from the PhD thesis of H. Strahl (H. Strahl (2007) “Kdp-dependent K^+ homeostasis of the halophilic archaeon *Halobacterium salinarum*”, PhD thesis, University of Osnabrück).
The plasmid encoding the full-length *kdp* promoter fused to *bgaH* (pDSK1) was taken from the diploma thesis of E. Özyamak (E. Özyamak (2005) “Charakterisierung des *kdpFABCcat3*-Promotors aus *Halobacterium salinarum* R1”, diploma thesis, University of Osnabrück).

Chapter III

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

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

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This chapter is published as:

Kixmüller D, Greie J-C (2012) Construction and characterization of a gradually inducible expression vector for *Halobacterium salinarum*, based on the *kdp* promoter. *Appl Environ Microbiol* 78:2100–2105
DOI: 10.1128/AEM.07155-11

Due to the publication procedure by a peer-reviewed journal is the published version a slight modified form of the thesis submitted for examination.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (grant GR 2698/1-1).

Chapter IV

An ATP-driven potassium pump promotes long-term survival of *Halobacterium salinarum* within salt crystals

An ATP-drivenpotassium pump promotes long-term survival of *Halobacterium salinarum* within salt crystals

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This chapter is published as:

Kixmüller D, Greie J-C (2012) An ATP-driven potassium pump promotes long-term survival of *Halobacterium salinarum* within salt crystals. *Environ Microbiol Rep* 4:234–241
DOI: 10.1111/j.1758-2229.2012.00326.x

Due to the publication procedure by a peer-reviewed journal is the published version a slight modified form of the thesis submitted for examination.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (grant GR 2698/1-1).

An dieser Stelle möchte ich den Personen, die mich während meiner Promotion begleitet haben, meinen herzlichen Dank aussprechen:

Ein besonderer Dank gilt meinem Doktorvater Prof. Dr. Karlheinz Altendorf, der mir die Möglichkeit gegeben hat, meine Dissertation unter hervorragenden Arbeitsbedingungen in einer tollen Arbeitsgruppe zu erstellen, mich stets in allen Belangen unterstützt hat und mir die Möglichkeit des fachlichen Austausches mit anderen Wissenschaftlern im Rahmen zahlreicher nationaler und internationalen Konferenzen gegeben hat.

Weiterhin bedanke ich mich bei Prof. Felicitas Pfeifer für die Begutachtung dieser Arbeit.

Bei meinem Projektleiter Dr. Jörg-Christian Greie möchte ich mich ausgesprochen herzlich für die wissenschaftliche Betreuung, aber auch für die Freiheit des selbstständigen und eigenverantwortlichen Arbeitens bedanken.
Für Deine Unterstützung und den Einsatz, insbesondere beim Abschluss dieser Arbeit, vor allem aber für Dein Verständnis und die ermutigenden sowie aufbauenden Worte in diversen Krisenzeiten bin ich Dir sehr dankbar.

Ein weiteres großes Dankeschön gilt meinem früherem Halo-Kollegen Henrik für sein beständig anhaltendes, bereitwilliges Mitdenken und die Diskussionsbereitschaft auch noch nach etlichen Jahren, die mir immer das Gefühl eines stetig Verbündeten gab.

Meinen weiteren Ex-Mitstreitern aus Labor 119 sowie der gesamten ehemaligen Arbeitsgruppe Mikrobiologie danke ich von Herzen für die wissenschaftliche Hilfe sowie die lustige und angenehme Atmosphäre auf unserem alten Flur. Hier seien besonders Ulla und Charlotte erwähnt, die mich im Halo-Projekt hilfreich unterstützt haben. Aber auch natürlich allen Anderen gilt: Danke für die tolle Zeit!

Ganz besonders danke ich Kerstin, Vera und Inga. Ihr habt mich in vielen Höhen und Tiefen begleitet, und mich immer, wenn wieder mal nötig, aufgemuntert. Besonders für die vielen wertvollen wissenschaftlichen Diskussionen aber auch für das alltägliche Geplauder danke ich Euch sehr. Die unnachahmliche, freundschaftliche Laboratmosphäre wird mir in bester Erinnerung bleiben.

Ein großes Dankeschön gebührt an dieser Stelle auch meiner Familie sowie dem Rest der Sippe und all meinen Freunden, die Anteilnahme an meinem Werdegang hatten. Eure außerordentliche Zuversicht und Unterstützung haben mich während der gesamten Doktorarbeit motiviert. Danke, dass Ihr immer für mich da seid und für Euer Verständnis, dass ich nicht so häufig den Weg in die Heimat gefunden habe, da am Wochenende mal wieder meine Bakterien „gefüttert“ werden mussten.

Zuletzt gilt mein Dank Christian, der mir immer zur Seite steht und mich mit all meinen Launen geduldig erträgt. Ich danke Dir für Deine Liebe, die manchmal notwendigen, tröstenden Worte und Deine alltägliche Unterstützung (mein Putzboy ☺), besonders in der Endphase meiner Arbeit.

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Since 05/2006	Doctoral thesis in biological science at the Department of Microbiology in the research group of Prof. Karlheinz Altendorf, University of Osnabrück Ph.D. thesis: "Transcriptional regulation and physiological importance of the *kdp*-system from the halophilic archaeon *Halobacterium salinarum*"
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Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise unentgeltlich geholfen.

1. Erste Informationen bezüglich der Bestimmung des Transkriptionsstarts und des potentiellen Terminators des *kdpFABCQ*-Transkripts sowie die Existenz eines Operators beteiligt an der Transkriptionsregulation des *kdpFABCQ* Operons wurden während meiner Diplomarbeit gewonnen (D. Kixmüller (2006) "Untersuchungen zur Promotorstruktur des *kdpFABCcat3*-Operons von *Halobacterium salinarum*", Diplomarbeit, Universität Osnabrück).
2. Die in dieser Arbeit gezeigten Studien zur globalen Transkriptionsantwort sowie die Analysen zur potentiellen Beteiligung der Zwei-Komponenten-Systeme aus *H. salinarum* an der transkriptionellen Regulation des *kdpFABCQ* Operon wurden von Dr. Henrik Strahl (2007) durchgeführt (H. Strahl (2007) "Kdp-dependent K^+ homeostasis of the halophilic archaeon *Halobacterium salinarum*", Dissertation, Universität Osnabrück).
3. Diverse Plasmide wurden wie aufgeführt während der Promotion von Henrik Strahl (2007), der Diplomarbeit von Ertan Özyamak (E. Özyamak (2005) "Charakterisierung des *kdpFABCcat3*-Promotors aus *Halobacterium salinarum* R1", Diplomarbeit, Universität Osnabrück) und der Bachelorarbeit von Charlotte Müller (C. Müller (2009) "Untersuchungen zur Spezies-spezifischen Expression des *kdp*-Operons aus *Halobacterium salinarum*", Bachelorarbeit, Universität Osnabrück) erstellt (alle Abteilung Mikrobiologie, Arbeitsgruppe Prof. Karlheinz Altendorf).

Weitere Personen waren an der inhaltlichen materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

________________________ (Ort, Datum)

________________________ (Unterschrift)