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Potassium uptake in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 mainly depends on a Ktr-like system encoded by *slr1509* (*ntpJ*)

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Abstract **The molecular basis of potassium uptake in cyanobacteria has not been elucidated. However, genes known from other bacteria to encode potassium transporters can be identified in the genome of *Synechocystis* sp. strain PCC 6803. Mutants defective in *kdpA* and *ntpJ* were generated and characterized to address the role of the Kdp and KtrAB systems in this strain. KtrAB is crucial for K^+ uptake, as the Δ*ntpJ* mutant shows slowed growth, slowed potassium uptake kinetics, and increased salt sensitivity. The Δ*kdpA* mutant has the same phenotype as the wild type even at limiting potassium, but a Δ*kdpA*Δ*ntpJ* double mutant is not viable, indicating a role of Kdp for potassium uptake when the Ktr system is not functioning.**

Key words: Ion transport; Salt stress; Sodium extrusion

1. Introduction

The complementary processes of sodium extrusion and potassium uptake are universal features of living cells, resulting in K^+ as the major intracellular cation. Available potassium concentrations in most environments are low, and all cells possess systems for energy-dependent potassium uptake. These different systems show homologies among each other and also to potassium channels [1]. In cyanobacteria a wealth of data exists on the phenomenology of potassium transport [2], but its molecular basis is not yet known. Here, combining genetic, physiological and biophysical techniques we addressed the potassium uptake routes in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (*Synechocystis* 6803 hereafter), making use of its completely sequenced genome [3].

In many bacteria, a Trk system is sufficient to accumulate potassium at high external concentrations. The constitutive Trk system in *Escherichia coli* has a low affinity for potassium (K_M = 1.5 mM). There is also a gene in the *Synechocystis* 6803 genome (*slr0773*) for a homologue of the K^+ transporter TrkA [4–6]. However, because of its low affinity it is no candidate for potassium uptake in *Synechocystis* 6803 during growth at micromolar K^+ concentrations.

A common high-affinity potassium pump of bacteria is the Kdp-ATPase, studied extensively in *E. coli* [1,7,8]. The four subunits are encoded by the *kdpFABC* operon in *E. coli*: KdpA binds potassium, and KdpB is the catalytic subunit which hydrolyzes ATP. The gene products of the *kdpDE* operon have a regulatory role. In *E. coli* Kdp is only expressed at low potassium concentration [4]. The genome of *Synechocystis* 6803 [3] harbors genes for the A, B, and C subunits of Kdp, which encode a well-conserved K^+-transport ATPase, while the regulatory histidine kinase KdpD is truncated and the corresponding response regulator KdpE is still not identified.

Another K^+ uptake system present in the genome of *Synechocystis* 6803 is the KtrAB system (also called KtrII) [9–12], with subunit A being encoded by open reading frame (ORF) *sll0493*, and the potassium-translocating subunit B being encoded by *slr1509*. The inducible KtrAB is found in several bacteria. It transports potassium by Na^+/K^+ symport, utilizes sodium motive force ($\Delta\mu(Na^+)$), and was studied intensively in *Enterococcus hirae*, where it is needed for growth at submillimolar potassium concentrations [12]. However, in some experiments K^+ uptake occurred without a sufficient $\Delta\mu(Na^+)$ [12], and the actual mechanism appears to be more complex than a simple symport; the implication of ATP or NADH in the operation of KtrAB has been suggested [10,12].

In *E. hirae* the gene for the B subunit, *ntpJ*, is part of the *ntp* operon encoding a V-type Na^+-ATPase. Therefore, it was initially assumed that the NtpJ protein is part of the sodium-translocating ATPase and the homologous gene *slr1509* in *Synechocystis* 6803 was initially assigned accordingly [3]. However, it was demonstrated that NtpJ = KtrB operates independently of the V-ATPase [12].

Here, we present the characterization of a mutant strain Δ*ntpJ* of *Synechocystis* 6803 with a disrupted gene for the potassium-translocating subunit of KtrAB, in comparison with a Kdp-ATPase-deficient mutant, which has a disrupted gene for the K^+-translocating subunit KdpA (*slr1728*). In addition, we also attempted to construct a double mutant defec-

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Abbreviations: DBMIB, 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; Km, kanamycin; WT, wild type; $\Delta\mu(Na^+)$, sodium motive force

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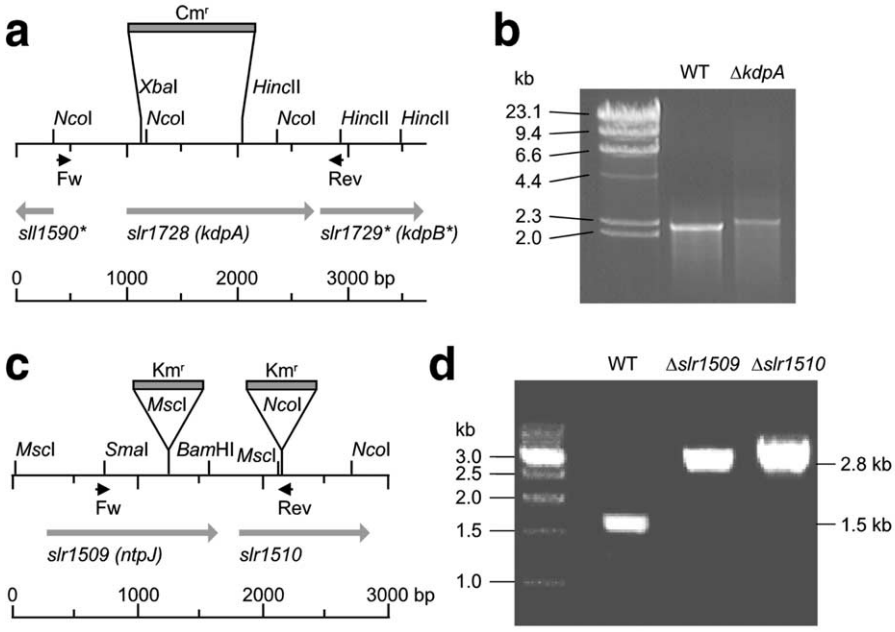

Fig. 1. Mutant genotypes and confirmation of segregation. a,c: Affected chromosomal regions of the WT and introduced drug resistance markers in the mutants Δ*kdpA* (a) and Δ*ntpJ* (c). Triangles indicate the binding positions of the PCR primers. b,d: PCR analysis of segregation for Δ*kdpA* (b) and Δ*ntpJ* (d).

tive in both *ntpJ* and *kdpA*, but it was impossible to segregate this strain.

2. Materials and methods

2.1. Growth conditions

Wild type (WT) and mutant cells of *Synechocystis* 6803 were grown at 30°C in liquid BG-11 medium [13], facultatively supplemented with 5 mM glucose as indicated, in aerobic batch cultures illuminated at 50 μE/m^2/s. Growth and cell density were monitored using the absorption of suspensions at 750 nm (OD_{750}). For growth of axenic cultures on plates 1.5% (w/v) Difco Bacto agar and 12.1 mM sodium thiosulfate were added to BG-11 medium. *E. coli* strains TG1 and DH5 were used for routine DNA manipulations [14,15].

2.2. DNA manipulation and construction of mutants

Total DNA of *Synechocystis* 6803 was essentially prepared according to [16,17]. The ORFs *slr1728* encoding KdpA, *slr1509* encoding NtpJ and *slr1510* were inactivated by interposon mutagenesis. Their coding sequences were amplified by polymerase chain reaction (PCR) with the following primers (KDP1: 5′-CCCAATGTACAGATAGCCGAGCA-3′; KDP2: 5′-GATTGAGCTTCACCCAGGCTTCC-3′; 1509/10-Fw: 5′-TCATTGCCACCACGCTAA-3′; 1509/10-Rev: 5′-TGGGAAATAAGGTGCGA-3′). The PCR products were cloned and drug resistance markers were introduced using suitable restriction sites (see Fig. 1 for details). Vectors containing the inactivated genes were used for transformation of *Synechocystis* 6803, and the mutated gene copies were integrated into the chromosome by homologous recombination. Segregation of the mutants took place by successive restreaking on plates with increasing drug concentrations. Segregation was confirmed by PCR using the primers described above (Fig. 1). Subsequent cultures of the segregated mutants were grown in the presence of 50 μg/ml chloramphenicol or 100 μg/ml kanamycin (Km). Descriptions were posted to CyanoMutants at http://www.kazusa.or.jp/cyano/Synechocystis/mutants/. Attempts to construct a double mutant defective in *kdpA* and *ntpJ* were undertaken, in which a completely segregated Δ*ntpJ* mutant was used as recipient. The Δ*ntpJ* gene was inactivated by introduction of a gentamicin resistance cartridge into its unique *Msc*I site. It was transformed with DNA of *kdpA* (*slr1718*), which was inactivated by insertion of a Km resistance cartridge into the unique *Eco*RI site. However, the obtained clones resistant to gentamicin and Km showed WT copies for *kdpA* after PCR analyses (20 clones tested). In a vice versa experiment, a segregated Δ*kdpA* strain was transformed by an inactivated *ntpJ* gene, again no completely segregated double mutant was obtained. Selection of clones occurred on BG11 medium supplemented with 4 mM potassium.

2.3. Determination of potassium concentration in cell extracts and culture medium

Potassium concentrations were determined by flame emission photometry using an AAS30 atomic absorption spectrophotometer (Analytik, Jena, Germany). Intracellular potassium was measured using silicone oil centrifugation [5]. Cell suspensions of 1.5 ml were centrifuged (13 000 rpm, 15 min) through 0.2 ml of low-viscosity silicone oil (Fluka DC 702 with 15% *n*-octane). The pellet was extracted with 1.5 ml of 10% (w/v) trichloroacetic acid (TCA) overnight at room temperature. After a second centrifugation, the potassium concentration in the TCA extract was measured. The amount of K^+ in 1 ml of TCA extract was normalized to the original cell density of the respective culture (determined as OD_{750}).

2.4. Determination of potassium uptake rate

The initial rate of potassium uptake immediately after the onset of actinic illumination of potassium-depleted cells was measured using a potassium-sensitive electrode (type 15-221-3000 from Mettler-Toledo, Switzerland) in a chamber with 4 ml sample volume at pH 8. Chlorophyll (Chl) *a* concentration was 15 μM; potassium chloride and further additions as indicated in the legends were added to the cell suspension immediately before the onset of illumination. Potassium-depleted cells were prepared by washing with 'potassium-free' K0-BG-11 medium, followed by resuspension in K0-BG-11, overnight dark incubation and washing again. In K0-BG-11 all potassium salts of normal BG-11 are replaced by sodium equivalents; the residual potassium concentration was about 2 μM. Results for all experiments are given as average ± S.D. from at least three measurements.

3. Results

3.1. Disruption of the kdpA and ntpJ genes

ORF *slr1728*, encoding KdpA in *Synechocystis* 6803, was interrupted by a chloramphenicol resistance cartridge (Fig. 1a). In another strain ORF *slr1509*, encoding the NtpJ protein, was disrupted using a Km resistance cassette (Fig. 1c). Fig. 1b,d shows that, according to PCR analyses, in both

mutants the respective WT genes have disappeared, proving that both mutants are completely segregated. Additionally, a segregated mutant defective in *slr1510* was obtained, which showed none of the phenotypic changes observed for the *ntpJ* mutant, ruling out that polar effects of the *ntpJ* mutation are responsible for the observed phenotypic changes (not shown). Interestingly, in another study a deletion mutant comprising *slr1507*, *slr1508*, and *slr1509* could not be completely segregated [18]. Obviously one of the genes upstream of *ntpJ* is essential for growth. Like here, a completely segregated single mutant in *ntpJ* was obtained by Shibata et al. [19]. We obtained no segregation for a double mutant with both ORFs *slr1728* and *slr1509* being disrupted by two different antibiotic cartridges even when prolonged cultivation at elevated potassium concentrations (initially 4 mM, afterwards 100 mM) was performed (not shown).

3.2. Cell growth under different salt conditions

At low potassium concentrations, cell growth is limited by the available amount of K^+; accordingly the growth rate of WT cells increased by about 65%, when the KCl concentration in the medium increased from 50 μM to 350 μM (Fig. 2a). The Δ*kdpA* mutant showed an identical curve within the range of error, indicating that potassium uptake functioned similarly as in WT. In contrast, the growth rate of the Δ*ntpJ* strain was both much lower and essentially independent of the potassium concentration within the range studied (Fig. 2a). The same picture emerges when the cell densities of stationary cultures are compared: in the presence of potassium con-

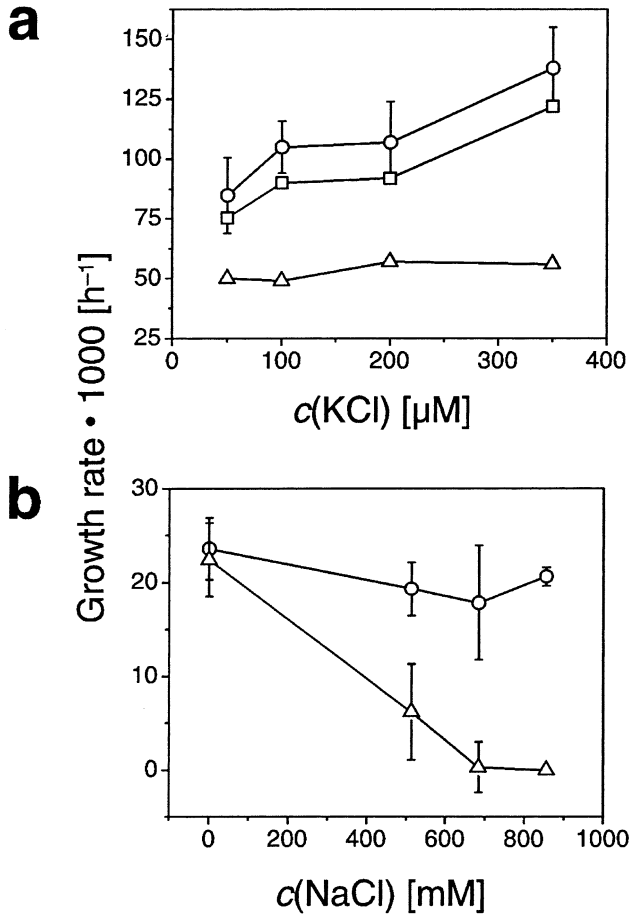

Fig. 2. Growth rate of WT (circles), Δ*kdpA* (squares) and Δ*ntpJ* (triangles) at different potassium (a) and sodium (b) concentrations. For a, K0-BG-11 medium supplemented with 5 mM glucose and KCl was used, for b BG-11 medium with added NaCl. For simplicity, a shows only error bars for WT; standard deviations for the other strains are similar. a: High growth rates were obtained at optimum laboratory conditions (with glucose, high light, low salinity). b: Slower growth rates were observed, when cultivation was performed without glucose, at lower light and in the presence of high salt.

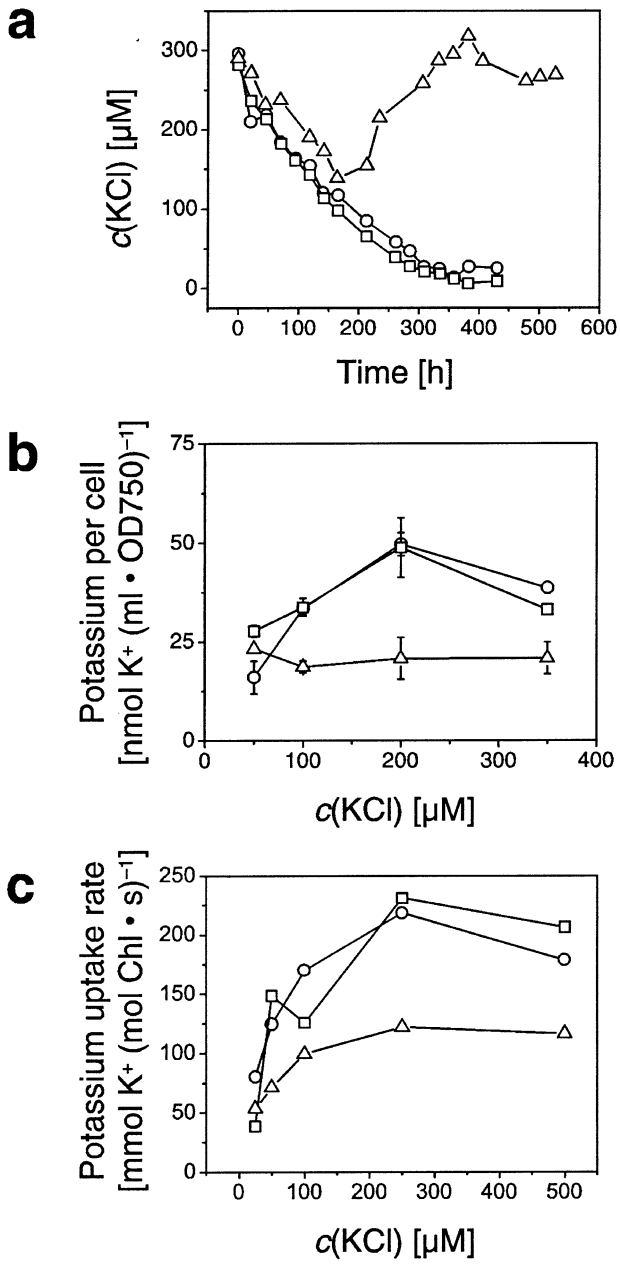

Fig. 3. Potassium accumulation of WT (circles), Δ*kdpA* (squares) and Δ*ntpJ* (triangles). a: Time course of free potassium in the culture supernatant, starting with 300 μM. b: Relative intracellular K^+ content of stationary cultures, in dependence on the initial concentration. c: Rate of light-induced potassium uptake of potassium-depleted cells, supplemented with KCl before illumination; the standard deviation is about ±35 mmol K^+/(mol Chl s). The rates were determined as initial rates immediately after the onset of illumination; the external potassium concentration declined rapidly and a steady state was reached within a few minutes. Therefore, the rates appear rather high and do not represent steady-state uptake rates.

centrations between 100 and 350 μM, both WT and Δ*kdpA* grew up to $OD_{750} = 3.7 \pm 0.5$, while Δ*ntpJ* only reached $OD_{750} = 2.9 \pm 0.5$, again indicating an inhibition of growth in this strain under low potassium conditions.

Additionally, the *ntpJ* mutant was unable to grow at high NaCl concentrations, which only slightly depressed the growth rate of the WT (Fig. 2b). The salt sensitivity of mutants affected in *ntpJ* has also been observed previously [18,19]. In contrast, no evidence was found for an increased salt sensitivity of the Δ*kdpA* mutant in our hands (data not shown) and in another study [18]. The Δ*ntpJ* strain also showed an increased sensitivity to osmotic stress, such as 5% (w/v) sucrose (not shown), but these effects were marginal compared to the inhibition of growth in the presence of NaCl.

3.3. Cellular uptake of potassium

During the growth of WT and Δ*kdpA* cultures under potassium limitation, the free potassium concentration declined continually, until virtually all potassium was sequestered (Fig. 3a). The Δ*ntpJ* strain showed a markedly different behavior: an initial decline of extracellular potassium, similar to the other two strains, was followed by an increase when growth of cells ceased, indicating a breakdown of potassium accumulation. The exact course of the external potassium concentration during growth of Δ*ntpJ* was rather irregular and difficult to reproduce; the curve in Fig. 3a is an example where almost all intracellular potassium is released back into the medium after the end of the exponential growth phase.

The potassium content on a per cell basis (Fig. 3b) showed a similar trend as the growth rates of the different strains (compare Fig. 2a): between 50 and 200 μM K^+ in the culture medium, there was a significant increase for both WT and Δ*kdpA* in the amount of intracellular potassium at stationary phase; for Δ*ntpJ* cells the values were much lower and independent of the available potassium amount.

While Figs. 2a and 3a,b indicated that the *efficiency* of potassium uptake was lower in the *ntpJ* mutant, kinetic measurements furthermore revealed that this corresponds to a lower *rate* of potassium uptake (Fig. 3c): in the saturation range above 200 μM, the rate of light-induced K^+ accumulation was about twofold higher in both WT and Δ*kdpA*. Kinetic measurements were also used to assess the effects of different inhibitors on light-induced K^+ uptake (Table 1).

Table 1
Effects of inhibitors on the rate of light-induced potassium uptake

Type of inhibitor	Relative potassium uptake rate in the investigated strains (%)		
	WT	Δ*kdpA*	Δ*ntpJ*
Control	100	100	100
Photosynthesis inhibitors			
50 μM DBMIB	−2 ± 1	−3 ± 3	2 ± 2
10 μM DCMU	47 ± 21	37 ± 8	48 ± 10
Potassium channel inhibitor			
100 μM TEA	61 ± 11	42 ± 6	84 ± 8
Sodium channel inhibitors			
100 μM DMA	37 ± 5	44 ± 13	103 ± 12
100 μM Lidocaine	60 ± 14	84 ± 22	110 ± 12
100 μM Phenytoin	62 ± 10	67 ± 14	108 ± 7
100 μM Quinidine	42 ± 12	51 ± 19	96 ± 8

Potassium uptake was measured using a potassium electrode in the presence of 50 μM KCl. The control rates were 125 ± 61 mmol K^+/(mol Chl s) (WT), 148 ± 63 mmol K^+/(mol Chl s) (Δ*kdpA*), and 71 ± 8 mmol K^+/(mol Chl s) (Δ*ntpJ*).

Two photosynthesis inhibitors, 3-(3,4-dichlorophenyl)-1,1′-dimethylurea (DCMU, blocks photosystem II) and 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB, blocks the cytochrome b_6f complex), had strong effects in all three strains, because the energy equivalents (ATP and NADPH) provided by the photosynthetic light reactions drive the light-dependent potassium uptake: DBMIB interrupts both linear and cyclic photosynthetic electron flow, and accordingly potassium uptake was completely suppressed. In contrast, in the presence of DCMU a residual activity of about 40% of the control rate was observed, because in the presence of this inhibitor some ATP synthesis via the photosystem I cycle is still possible. The potassium channel blocker tetraethyl ammonium (TEA) [20] inhibited all strains to a certain extent; however, in principle it may act on any route of potassium uptake (see Fig. 4) and is obviously rather unspecific. Four different inhibitors of sodium channels [20,21] inhibited potassium transport in both WT and Δ*kdpA*; in particular, 5-(*N*,*N*-dimethyl)-amiloride (DMA) and quinidine were highly effective. In striking contrast, all four compounds had no effect in the Δ*ntpJ* strain.

4. Discussion

Fig. 4 summarizes the available information on potassium transport in *Synechocystis* 6803, based on literature data, the experimental results of this paper and phylogenetic comparisons. The three K^+ transporters that can be identified in the genome are shown. In addition, there exist at least three potassium channels (*slr1575*, *sll0536*, *sll0993*, denoted KchX), one of which resembles the K^+ channel KefC from *E. coli* [9].

The data in Figs. 2a and 3a–c demonstrate a central role of NtpJ in potassium uptake in *Synechocystis* 6803, similar to *E. hirae* [10–12]. The physiological results are supported by the close similarity of the NtpJ protein from *Synechocystis* 6803 to well-characterized KtrB proteins from enterobacteria. Obviously, the membrane subunits of Ktr systems are phylogenetically related to corresponding proteins of the low-affinity Trk systems known from enterobacteria (Fig. 4b). This homology to well-characterized potassium transporters and the defect in potassium uptake of the mutant make it unlikely that NtpJ is directly related to a hypothetical Na^+-ATPase in *Synechocystis* 6803. The phylogenetic analysis also suggests that one soluble subunit of the Ktr system of *Synechocystis* 6803 is encoded by *sll0493* (Fig. 4b).

In *E. hirae*, uptake of potassium is coupled to sodium influx, and the required sodium gradient is provided by a V-type Na^+-ATPase [10]. We speculate that in *Synechocystis* 6803 the $\Delta\mu(Na^+)$ is generated instead by the sodium/proton antiporters NhaS, known to be the major transporters for sodium extrusion in cyanobacteria [22–24]. In *Synechocystis* 6803 there are five genes, *nhaS1* to *nhaS5* [22,23], with NhaS3 being of major importance for high salt tolerance [18]. Na^+/H^+ antiport is energized by proton pumping at the plasma membrane, which in turn depends on energy equivalents from photosynthesis [25], thus explaining the indirect action of photosynthesis inhibitors on potassium uptake (Fig. 4a). There are also some hints for a direct ATP-dependent Na^+ transport in *Synechocystis* 6803 [26], but this has not yet been experimentally verified. Accordingly, a sodium ATPase, functionally complementing KtrAB in *E. hirae*, is depicted only in gray in Fig. 4a. The inhibition of potassium uptake by sodium chan-

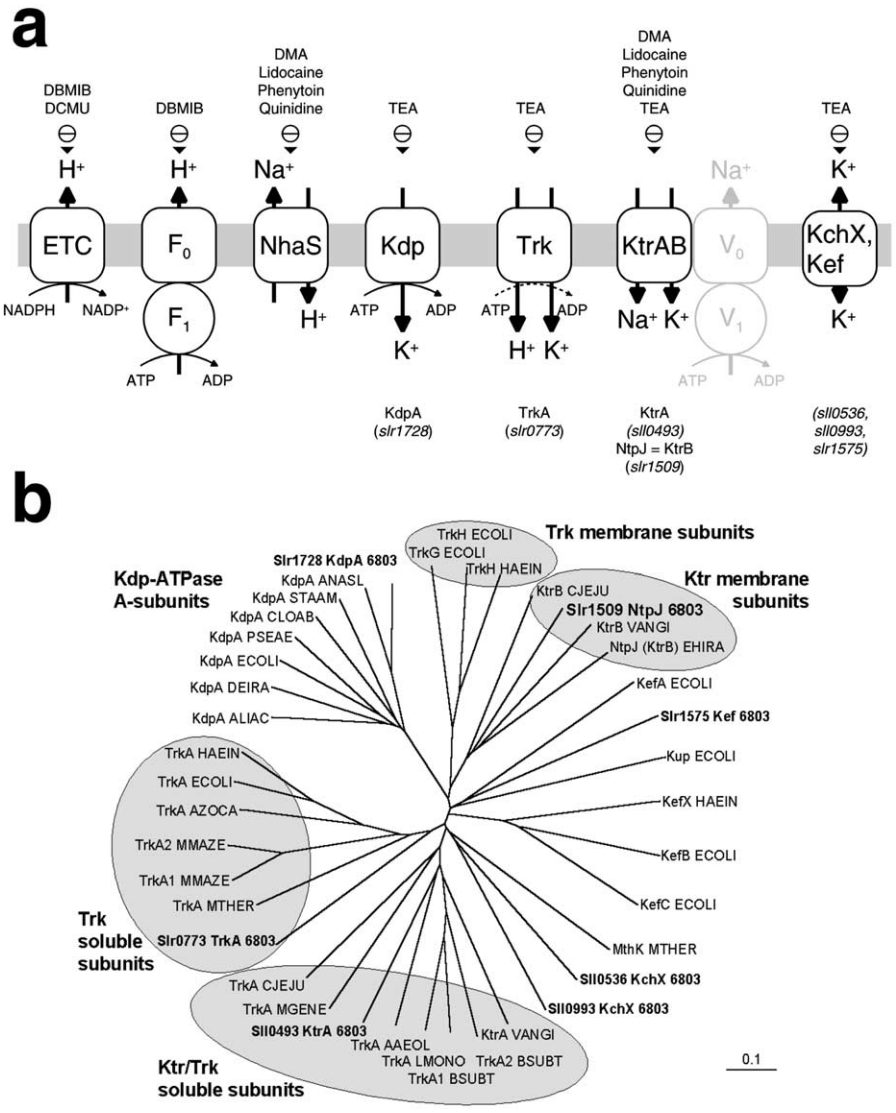

Fig. 4. a: Genes and proteins implicated in potassium uptake in *Synechocystis* 6803, with possible sites of inhibitor action. ETC = electron transport chain. In the Trk system ATP is only a regulator, indicated by a dashed arrow. b: Unrooted phylogenetic tree (ClustalX) showing the relation of putative potassium transport proteins encoded in the *Synechocystis* 6803 genome [3] (bold) to related proteins found in data bases. For the phylogenetic comparison some of the most similar proteins found after Blast comparisons [28] were selected. Preference was given to functionally characterized proteins from enterobacteria.

nel blockers [20,21] in WT and Δ*kdpA* (Table 1) could be due either to direct inhibition of NtpJ, to indirect inhibition of the NhaS, or to both. In any case, the lack of effect of sodium channel inhibitors in Δ*ntpJ* clearly demonstrates that KtrAB is the only significant Na^+-dependent K^+ uptake system in *Synechocystis* 6803.

The data presented here show a marked salt sensitivity in the *ntpJ* mutant, in accordance with previous reports [18,19]. In *E. hirae* it has been experimentally shown that the Ktr system employing a close homologue to NtpJ plays a role in potassium uptake coupled to sodium influx, rather than in sodium efflux [10]. Thus, a direct function of NtpJ in sodium extrusion as previously speculated [18,19] is quite unrealistic, as in this case the potassium uptake against the electrochemical gradient would be coupled with sodium extrusion against the $\Delta\mu(Na^+)$. We conclude that the salt sensitivity of the *ntpJ* mutant probably results from a decreased potassium accumulation, which is a prerequisite for high salt tolerance [23]: the outward sodium gradient across plasma membranes has to be accompanied by an inward potassium gradient, and deletion of NtpJ, directly affecting K^+ accumulation, may indirectly also impede Na^+ extrusion.

The absence of a phenotype for the *Synechocystis* 6803 Δ*kdpA* mutant even at limiting potassium concentrations is surprising, as Kdp in *E. coli* is an emergency system expressed only under potassium starvation [4], and a Kdp-deficient strain of *E. coli* cannot grow at micromolar potassium concentrations [7]. However, the fact that a *Synechocystis* 6803 double mutant lacking both KdpA and NtpJ is not viable indicates a contribution of the Kdp system under conditions where KtrAB is not operating. It should be noted that KtrAB is dependent on a sodium gradient (i.e. high sodium outside), but Kdp is not. *Synechocystis* 6803, which can live in a wide range of salinities, may utilize either system in a flexible way, in response to the external sodium concentration. Obviously, the Kdp system is responsible for the remaining potassium

uptake activity in the Δ*ntpJ* strain, which is however not sufficient to support growth rates comparable to the strain in which only the Ktr system operates (Δ*kdpA* mutant). Recently it has been shown that a truncated KdpD protein from *Anabaena* could be combined with the C-terminal part of *E. coli* KdpD leading to a functional histidine kinase able to phosphorylate *E. coli* KdpE in vitro [27]. Obviously, the regulatory proteins of *kdp* are differently organized in cyanobacteria compared to *E. coli*, while the structural subunits of the Kdp-ATPase are well conserved.

As shown in Fig. 4, there is also genetic evidence for a third potassium uptake system, Trk, in *Synechocystis* 6803, which is dependent on both the proton gradient and ATP [4–6]. However, Trk alone is not sufficient for potassium uptake in *Synechocystis* 6803, as demonstrated by the failure to segregate in the Δ*kdpA*Δ*ntpJ* double deletion strain even in the presence of millimolar potassium.

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