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| 学位論文題目 | Elucidation of Novel Functions of $K^+$ Transporters Involved in Survival of *Escherichia coli* Under Potassium Limited Condition<br>(大腸菌の生存に関与する $K^+$輸送体の新規機能の解明) |
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# 論文内容要約

**Background**

Potassium ion ($K^+$) has many essential functions that contribute to the normal functioning of the cell, such as maintaining pH homeostasis, membrane potential formation, and osmoregulation[1]. Therefore, *Escherichia coli* has multiple $K^+$ transport systems on its inner membrane to keep a high concentration of intracellular $K^+$. The major $K^+$ uptake systems in *E. coli* is Kup, Kdp, and Trk. Each system has a different characteristic to support *E. coli* growth under various conditions: 1) Kdp is a high-affinity $K^+$ transporter, and it only expressed during $K^+$ starvation[2]; 2) Kup is unique because of its ability to uptake $Cs^+$ beside $K^+$ and $Rb^{+3}$; 3) *E. coli* Trk-type $K^+$ transport system consists of two different pathways: TrkG and TrkH, which are homolog proteins[4].

It has been reported that, under $K^+$ limitation, the cell has an ability to use other alkali metal cation to substitute $K^+$ to support growth[5,6]. This study focuses on two possible $K^+$ substitutes that are not commonly used or accumulated in *E. coli* cell; they are cesium ion ($Cs^+$) and sodium ion ($Na^+$). $Cs^+$ is relatively rare in the environment and generally considered harmful for living organisms while $Na^+$ is toxic when present at high concentration in the cytosol. It has been reported that $Cs^+$ can enter the *E. coli* cell via one of the major $K^+$ transport system, Kup[3]. Meanwhile, the $Na^+$-dependent $K^+$ uptake activity has been reported from some relatives of *E. coli* Trk $K^+$ transporters, such as wheat HKT1[7] and *Synechocystis* KtrABE[8]. Therefore, the objective of this research is to examine the possibility of $Cs^+$ and $Na^+$ as $K^+$ substitute under low $K^+$ condition and examine how both cations influence $K^+$ transport systems in *E. coli*.

**Method**

Growth test: *E. coli* BW25113 (wild type) or its derivatives mutants were grown in minimal medium (46 mM $Na_2HPO_4$, 23 mM $NaH_2PO_4$, 8 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 6 mM $FeSO_4$, 10 μg/ml thiamine and 1% glucose) supplemented with 30 mM KCl (or otherwise indicated). The grown culture was harvested and washed with K0 buffer (46 mM $Na_2HPO_4$, 23 mM $NaH_2PO_4$, 8 mM $(NH_4)_2SO_4$), then resuspended with the same buffer. The cell suspension was inoculated into the new medium with various concentration of $K^+$ or $Cs^+$. The cultures were incubated at 30 °C, 150 rpm for either 15 or 24 hrs. The cell growth was measured by $OD_{600}$. For $Na^+$-free medium, we used phosphoric-acid based medium (modified from previously described defined medium[9]) that contains 8 mM $H_3PO_4$, 8 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 6 μM $FeSO_4$, 29 μM $MnSO_4.5H_2O$, 12 μM $CaCl_2.2H_2O$, 17 μM

$ZnSO_4.7H_2O$, 0.4 µM $CuSO_4.5H_2O$, 0.4 µM $CoCl_2.6H_2O$, 0.73 µM p-aminobenzoic acid, 4.9 µM pyridoxine HCl, 1.5 µM thiamine, 1 % (w/v) glucose, 0.2 - 3.0 mM amino acid mixtures, and additional KCl and NaCl (pH 7.4 adjusted by Tris).

Cation uptake experiments: This protocol was conducted as described previously[8] with some modifications. *E. coli* was grown in the minimal medium supplemented with 20 mM KCl, supplemented with 50 µg/mL ampicillin and 0.1 mM IPTG, if necessary. The full-grown culture was harvested and treated by 1 mM EDTA in 120 mM Tris HCl pH 8.0. The treated cell was washed two times with buffer (50 mM Tris-HCl or 200 mM HEPES NaOH or 200 mM HEPES Triethanolamine pH 7.50) and resuspended with the same buffer. The cation uptake was performed in the cell suspensions with $OD_{570}$ of 3. 10 mM of glucose was added before the $K^+$ addition, $K^+$ was added at indicated final concentration. At the designated time points, 1 mL of samples from were taken and centrifuged through silicone oil (Sigma Aldrich) to separate the buffer and cells. The buffer was removed, and the cell was disrupted by 5 % trichloroacetic acid and heated 100 °C for 5 minutes. The $K^+$ content was determined using an atomic absorption spectrometer (iCE 3500 Thermo Fisher Scientific AA Spectrometer), and the total protein content in the cell pellets was measured by using BCA protein assay (Thermo Scientific).

**Part 1: $Cs^+$ acts as a nutrient under low $K^+$ condition**

This part of this research has been published: Tanudjaja, E., Hoshi, N., Su, Y.-H., Hamamoto, S. & Uozumi, N. Kup-mediated $Cs^+$ uptake and Kdp-driven $K^+$ uptake coordinate to promote cell growth during excess $Cs^+$ conditions in *Escherichia coli*. *Sci. Rep.* 7, 2122, doi: 10.1038/s41598-017-02164-7 (2017). Link to the publisher version http://www.nature.com/articles/s41598-017-02164-7

$Cs^+$ and $K^+$ classified as the same alkali metal cation group, which imply a chemical similarity between those two. Therefore, despite its toxicity, it might be possible for *E. coli* to use $Cs^+$ as $K^+$ substitute for growth under a particular condition. The growth of *E. coli* wild type in minimum medium supplemented with 25 mM $Cs^+$ exhibited significantly lower cell growth than that of supplemented by the equimolar concentration $K^+$; This result indicates that $Cs^+$ cannot substitute $K^+$ completely as a nutrient for growth. However, the combination of low $K^+$ (0.1 mM $K^+$) and additional supplementation of $Cs^+$ up to 25 mM improved the *E. coli* growth approximately 3.17 times higher compared to the control medium (without $Cs^+$). This result indicates that $Cs^+$ can be used as a nutrient for *E. coli* growth under $K^+$ limited condition.

The previous study has exhibited the $Cs^+$ uptake ability of Kup[3]. Our result demonstrates that the deletion of *kup* reduced the cell growth on the low-$K^+$ and high $Cs^+$ medium, mainly due to the reduction of accumulated $Cs^+$ inside the cell. However, the cation uptake experiment on *E. coli* LB2003, which lack of the three major $K^+$-transporters, overexpressing Kup showed that the high concentration of $Cs^+$ uptake led to $K^+$ extrusion from the cell. These results imply that other $K^+$ transporters may also be needed to maintain the basal $K^+$ intracellular. The growth test on individual single knockout $K^+$ transporter mutants revealed that growth of both *kup* and *kdp* mutants was severely affected in the low-$K^+$ high-$Cs^+$ medium (0.1 mM $K^+$ and 25 mM $Cs^+$). The growth of *kup* and *kdp* mutants was inhibited by 29 % and 93 % respectively compared to the wild type. These data indicate that Kdp and Kup play a crucial role in the *E. coli* growth under low-$K^+$ and high-$Cs^+$ condition. We propose that the activity of Kdp, which is the high-affinity $K^+$ transporter, fulfill the basal $K^+$ requirement while $Cs^+$, transported by Kup, works as a substitute cation, and improves the growth. In summary, these findings reveal that $Cs^+$ can act as a nutrient and

enhances the *E. coli* growth under limited $K^+$ condition, assisted by Kup and Kdp transporters[10].

**Part 2: $Na^+$ is transported by Trk type $K^+$ transport system**

TrkG and TrkH belong to a Trk/Ktr/HKT family transporter that presents not only in the bacteria but also in fungi and plant[11]. Even though they classified as $K^+$ transporters, some of them are $Na^+$-activated $K^+$ transporter (*Synechocystis* KtrABE[8]), $K^+$-$Na^+$ co-transporter (wheat HKT1[7]) or even a $Na^+$-transporter (*Arabidopsis thaliana* HKT1[8]). Considering those transporters are evolutionarily related to *E. coli* Trk-type transporters; there is a possibility that $Na^+$ also involves in the $K^+$ transport activity of *E. coli* TrkG or TrkH.

In order to study the sole $K^+$-uptake activity of TrkG or TrkH without the interference of the other $K^+$ uptake systems, the triple knockout $K^+$ transporter mutants Δ*dhu* (Δ*kdpA trkH kup*) and Δ*dgu* (Δ*kdpA trkG kup*) were constructed, respectively. The $K^+$ uptake experiments on a high-$Na^+$ buffer (HEPES NaOH) and $Na^+$-free buffer (HEPES Triethanolamine) showed that the presence of $Na^+$ in the buffer significantly enhanced the $K^+$ transport activity of TrkG but not TrkH. The $K^+$ uptake of TrkH was relatively similar on both buffers. These results imply that $Na^+$ activates TrkG-mediated $K^+$ uptake. The $Na^+$ activation of $K^+$-uptake activity of TrkG was specific as the additions of other cations ($Rb^+$, $Li^+$, $Cs^+$, and $Ca^{2+}$) were unable to produce the same activating effect on TrkG. Those results suggest that TrkG is a $Na^+$-dependent $K^+$ uptake system while TrkH is a $Na^+$-independent $K^+$ uptake system.

Some $Na^+$-dependent $K^+$-uptake Trk/Ktr/HKT transporters (e.g., *Vibrio alginolyticus* KtrB[12]) also mediate $Na^+$ transport while others (e.g., *Synechocystis* KtrB[8]) do not transport $Na^+$. To examine the $Na^+$ uptake activity of TrkG, the $Na^+$ uptake experiment was performed on *E. coli* TO114 (Δ*nhaA nhaB chaA*) overexpressing TrkG. It shows higher accumulation of $Na^+$ compared to the cell expressing empty vector nor TrkH. This result indicates the $Na^+$ uptake activity of TrkG. Moreover, we have confirmed the $Na^+$ uptake activity of TrkG using the quadruple knockout mutant of Δ*abcg* (Δ*nhaA nhaB chaA trkG*). The $Na^+$ accumulation of this quadruple mutant was significantly lower compared to its parental strains, Δ*abc* (Δ*nhaA nhaB chaA*), proving that TrkG plays a role in $Na^+$ uptake. Taken together, these results confirmed that $Na^+$ not only activate TrkG-mediated $K^+$ uptake but also is transported into the cell via TrkG.

TrkG and TrkH are homolog proteins with similar predicted structure and 41% amino acid identity[4]. However, only one out of four TrkG selectivity filter was identical to TrkH. We tried to determine which part of TrkG that is responsible for its $Na^+$ dependency by constructing various combinations of TrkG-TrkH chimera proteins and selective pore variants. Unfortunately, all the variant lost its $K^+$ uptake ability and the critical part responsible for $Na^+$ dependency of TrkG could not be identified. However, the domain-0 (D0) of TrkG might be essential for its expression and function as the truncation of D0 on TrkG protein leads to the expression failure.

The utilization of $Na^+$ as $K^+$ substitution under low $K^+$ condition has been observed in plants[13,14]. $Na^+$ is most likely to be used to replace the unspecific functions of $K^+$, such as maintaining osmotic pressure[13]. We examined the possibility of $Na^+$ to enhance the *E. coli* growth under low $K^+$ condition. The growth test of *E. coli* wild type was performed in phosphoric acid-based medium supplemented by low $K^+$ (0.01 mM $K^+$) and various $Na^+$ concentration. It did not show any significant improvement in cell growth. However, when the growth test was performed on the $Na^+$-efflux-transporters mutant, Δ*abc* (Δ*nhaA nhaB chaA*), the additional supplementation of $Na^+$

up to 25 mM into the low $K^+$ medium (0.01 mM $K^+$) improved the growth approximately 3.2 times compared to the control medium (without $Na^+$). These results suggested that $Na^+$ cannot efficiently improve *E. coli* growth as it naturally prefers to export $Na^+$ outside the cell; therefore, the positive effect of $Na^+$ on cell growth was difficult to be observed in the wild type.

## Conclusions

In summary, this study demonstrates that $Cs^+$ can be used as a nutrient for *E. coli* under $K^+$ limited conditions, but it requires the coordination of both high-affinity $K^+$-uptake mediated by Kdp and $Cs^+$-uptake by Kup. On the other hand, $Na^+$ might not directly enhance the growth of *E. coli* wild type under low $K^+$ conditions, but $Na^+$ influences the Trk-type $K^+$ transporters activity differently; The presence of $Na^+$ activates $K^+$-uptake activity mediated by TrkG but not TrkH.

This research gives some novel features regarding the *E. coli* $K^+$ transport systems: 1) $Cs^+$-uptake mediated by Kup improves cell growth under low $K^+$ condition; 2) Kdp-mediated $K^+$ uptake activity is crucial to sustaining growth under low $K^+$ and high $Cs^+$ condition; 3) TrkH is $Na^+$-independent $K^+$ uptake transporter; 4) TrkG is $Na^+$-dependent $K^+$ uptake transporter and shows $Na^+$ uptake activity.

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