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Heterologous Expression of a Putative K^+/H^+ Antiporter of *S. coelicolor* A3(2) Enhances K^+, Acidic-pH Shock Tolerances, and Geldanamycin Secretion

Song, Jae Yang[1], Young Bin Seo[2], Soon-Kwang Hong[3], and Yong Keun Chang[2]*

[1]*Bio Lab, Energy R&D Center, SK Innovation Global Technology, Daejeon 305-712, Korea*
[2]*Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea*
[3]*Division of Bioscience and Bioinformatics, Myung-Ji University, Yongin 449-728, Korea*

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Heterologous expression of a putative K^+/H^+ antiporter of *Streptomyces coelicolor* A3(2) (designated as *sha4*) in *E. coli* and *Streptomyces hygroscopicus* JCM4427 showed enhanced tolerance to K^+ stress, acidic-pH shock, and/or geldanamycin production under K^+ stress. In a series of K^+ extrusion experiments with *sha4*-carrying *E. coli* deficient in the K^+/H^+ antiporter, a restoration of impaired K^+ extrusion activity was observed. Based on this, it was concluded that *sha4* was a true K^+/H^+ antiporter. In different sets of experiments, the *sha4*-carrying *E. coli* showed significantly improved tolerances to K^+ stresses and acidic-pH shock, whereas *sha4*-carrying *S. hygroscopicus* showed an improvement in K^+ stress tolerance only. The *sha4*-carrying *S. hygroscopicus* showed much higher geldanamycin productivity than the control under K^+ stress condition. In another set of experiments with a production medium, the secretion of geldanamycin was also significantly enhanced by the expression of *sha4*.

Key words: K^+/H^+ antiporter, salt tolerance, pH shock tolerance, geldanamycin

A major challenge of soil bacteria, plants, and human pathogens that live in a harsh habitat is to have resistance to high salt concentration, extreme pH, and/or drought [1, 10, 13, 20, 26, 41]. Many of them have sophisticated cation transporter systems, such as the Na^+/H^+ antiporter and K^+/H^+ antiporter, for their survival [1, 13, 29, 38, 42]. It is demonstrated that the cation/H^+ antiporter has a function of intracellular cation and pH regulations [8], osmolarity regulation [36, 38], Li^+ efflux [14, 30], and cell volume regulation [9, 15, 25]. They are also known to provide the host with a resistance to oxidative stress [18], cold shock [33], or drought [6, 40], suggesting that they play important roles in cell homeostasis under various stress conditions.

The most well studied cation/H^+ antiporters in *E. coli* are Na^+/H^+ antiporters (NhaA, NhaB, and ChaA) and K^+/H^+ antiporters (KefB and KefC). NhaA, NhaB, and ChaA have a primary role in sodium extrusion and pH regulation [29, 31, 35]. It is reported that KefB and KefC protect the host against DNA damage and regulate intracellular pH.

Recently, a number of intensive studies on the functions of the Na^+/H^+ and K^+/H^+ antiporters were performed. Deletion of Na^+/H^+ antiporter genes of *Bacillus subtilis* caused a higher Na^+ sensitivity than that of the control [41]. Heterologous expression of *nhaA* of *E. coli* improved salt and drought tolerances in rice [40]. Heterologous expression of the Na^+/H^+ antiporter of *Arabidopsis* (AtNHX) increased salt tolerance of Na^+-sensitive yeast strains [13]. Overexpression of the Na^+/H^+ antiporter from a halotolerant cyanobacterium, *Synechococcus* sp. PCC 7942, drastically improved salt tolerance [39]. In addition, it was demonstrated that biomolecules secretion was facilitated by the activity of the Na^+/H^+ antiporter [16]. In another case, it was found that Na^+/Ca^{2+} and Na^+/H^+ antiporters were involved in the process of prolactin secretion in anterior pituitary cells [34]. These results suggested that the Na^+/H^+ antiporter was involved in many biological processes including pH regulation, biomolecules secretion, and promotion of secondary metabolism.

Streptomyces coelicolor A3(2), the microorganism used in this study, is a soil filamentous bacterium that produces versatile biologically active compounds [3]. It has complicated morphological differentiation and secondary metabolism, which are regulated by a number of environmental signals including nutrient stature [7], pH [17, 21, 22, 24, 37], and

**Corresponding author*
Phone: +82-42-350-3927; Fax: +82-42-350-3910;
E-mail: ychang@kaist.ac.kr

temperature [11]. The cation transporter systems in *S. coelicolor* A3(2) are believed to have important roles in changes of cell morphology and the regulation of intracellular pH. Eight putative cation/H^+ antiporters in *S. coelicolor* A3(2) are reported in the NCBI database. In the previous works by our group, these eight putative cation/H^+ antiporters were cloned and expressed in *Streptomyces lividans* TK24 [23]. It was found that a transformant with one of the cation/H^+ antiporters showed a more significant medium pH fluctuation and a higher actinorhodin productivity than the control. Among those eight putative cation/H^+ antiporters, SCO3185 (designated as *sha4*) and SCO7832 (designated as *sha8*) showed the most prominent results. Interestingly, the construction of a *sha4*-deletion strain could not be accomplished despite a number of trials, indicating the role of *sha4* might be critical and indispensable for cell survival.

In this study, we expressed *sha4* in *E. coli* derivatives partially lacking in the K^+/H^+ antiporter system to investigate its roles in cation efflux activity, enhancement of pH shock, and salt tolerance. In addition, *sha4* was introduced to an industrial strain of *S. hygroscopicus* JCM4427 for the evaluation of its capability of enhancing geldanamycin productivity under K^+ stress.

Materials and Methods

Strains and Plasmids

The gene *sha* was heterologously expressed in *E. coli* for the identification of its functions, and it was heterologously expressed in *Streptomyces hygroscopicus* for the elucidation of its effects on geldanamycin production. Mutant strains derived from *E. coli* K12 (ATCC 27325) and *S. hygroscopicus* JCM4427 (Japanese Culture Collection of Microorganism) were constructed and then used. *E. coli* DH5α was used for routine DNA subcloning. Plasmids of pGEM-T (Promega, USA) and pKC1139 were used as cloning and expression vector, respectively. The plasmid pRedET for Red/ET recombination was purchased from Gene Bridges (Heidelberg, Germany) and was transformed into *E. coli* K12 for the construction of *E. coli* strains deficient in the K^+/H^+ antiporter. *E. coli* ET12567/pUZ8002 was used as a donor strain for intergeneric conjugation of *E. coli*–*Streptomyces*. Characteristics of these strains and plasmids are listed in Table 1.

Medium and Growth Conditions

Liquid LB or LBK (LB with KCl instead of NaCl) was used for *E. coli* cultures. *S. hygroscopicus* JCM 4427 was grown on cellophane-covered agar plates containing a geldanamycin production medium [37]. When necessary, a salt-free medium was prepared by omitting KCl addition. For K^+ stress experiments, each medium was supplemented with various concentrations of KCl. For pH-shock stress experiments, an appropriate amount of 1 N of HCl or NaOH solution was applied to the growing cells at an OD of 0.5.

When necessary, apramycin (50 μg/ml), ampicillin (50 μg/ml), kanamycin (15 μg/ml), erythromycin (160 μg/ml), chmoramphenicol (20 μg/ml), and nalidixic acid (50 μg/ml) purchased from Sigma Aldrich were used. Cultures were performed at 37°C for *E. coli* and at 28°C for *S. hygroscopicus* strains.

Construction of K^+/H^+ Antiporter (*kefB*)-Deletion *E. coli* Strains

Deletion of *kefB* was performed by using Red/ET recombination. A 1.75 kb neomycin cassette flanked by homology arms of *kefB* was generated by using a primer set (kefB-F: 5'-atggaaggttccgattttttactc

Table 1. Strains and plasmids used in this study.

Designation	Relevant characteristics	Source or Reference
Strains		
S. hygroscopicus JCM4427	Geldanamycin producer	JCM 4427
S. hygroscopicus pKC1139	JCM4427 carrying pKC1139	This study
S. hygroscopicus pSha4	JCM4427 carrying pSha4	This study
E. coli DH5α	supE44Δ*lac*U169(Ø80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*96 *thi*-1 *rel*A1	RBC bioscience
E. coli ET12567/pUZ8002	Donor strain of *E. coli*–*Streptomyces* conjugation with DNA methylation-deficient, kan^r, cmp^r	[9]
E. coli K12 derivatives		
K12C	*E. coli* K12 carrying a pKC1139, apr^r	This study
K12S4	*E. coli* K12 carrying a pSha4 , apr^r	This study
KKC	Δ*kefB*::neo carrying pKC1139, neo^r, apr^r, kan^r	This study
KKS4	Δ*kefB*::neo carrying pSha4, neo^r, apr^r, kan^r	This study
Plasmids		
pGEM	pUC19 backbone, T-cloning flank, amp^r	Promega
pRedET	Red/ET expression plasmid, amp^r	Gene Bridiges
pKC1139	*E. coli*–*Streptomyces* shuttle vector, apr^r	[4]
pSha4	*sha4*-carrying pKC1139, apr^r	This study

Abbreviation: Apr, apramycin; Cmp, chloramphenicol; Kan, kanamycin; Em, Erythromycin.

gcaggagtgctgtttctcttcgcggcaattaaccctcactaaagggcg-3'; kefB-R: ctactc aaattcatcccagccgtccagctggcgtcgttcttgttgcattttaatacgactcactctagggct) for Δ*kefB* construction. pRed/ET plasmid transformation into *E. coli* K12 and Red/ET recombinase protein induction were performed by following the manufacturer's instruction (Gene Bridges). For the selection of the Δ*kefB* transformant, the cultures were incubated for 3 h at 37°C and spread on a LB agar plate supplemented with kanamycin (15 µg/ml). Verification of Δ*kefB* was performed by PCR analysis.

Construction of *sha4* Transformants

Genomic DNA of *Streptomyces coelicolor* A3(2) (ATCC BAA471) was isolated according to the description by Kieser *et al.* [19] and was used for the PCR template. A putative K^+/H^+ antiporter (SCO3185; *sha4*) was amplified by using the following primers with different restriction sites: sha4-F (5'-ggatccagaaagcgtcaagagttagg-3', underline indicates *Bam*HI site) and sha4-R (5'-tctagattcagacagaggtggttcga-3', underline indicates *Xba*I site). The PCR product was then separated by electrophoresis with a 0.8% agarose gel. The DNA band excised from the agarose gel was purified by using a gel extraction kit (Qiagen, USA). The purified PCR product was ligated into pGEM t-vector and the recombinant plasmid was transformed into *E. coli* DH5α. The recombinant plasmid was harvested by using a mini plasmid preparation kit (INtron Biotech., Korea) and subsequently sequenced and analyzed. The sequenced plasmid was digested with *Bam*HI and *Eco*RI, and then 1.75 kb of *sha4* fragment was ligated into 6.5 kb pKC1139 by using T4 DNA ligase (Promega) for the construction of 8.25 kb of *sha4* expression vector (designated as pSha4). The pSha4 was transformed into the wild type of *E. coli* K12 and Δ*kefB* strains by electroporation at 1,350 V, 15 µF, and 600 Ω, and the transformants were selected by spreading on a LB plate supplemented with 50 µg/ml of apramycin.

Intergeneric conjugation between *E. coli* ET12567/pUZ8002 and *S. hygroscopicus* JCM4427 was performed following a published method [12] with slight modifications, for the construction of *sha4*-carrying *S. hygroscopicus* JCM4427.

Analysis

To determine K^+ extrusion activity of pSha4-carrying *E. coli* strains, concentrations of intracellular $[K^+]$ were measured by using an atomic absorption spectrophotometer [28]. Cell viability was determined by measuring colony forming units (CFU) [5]. Salt stress tolerance was determined by monitoring cell growth.

Salt and pH shock stresses tolerance of pSha4-carrying *S. hygroscopicus* JCM4427 was also determined by monitoring cell growth in surface-grown cultures supplemented with 400 mM of KCl. Measurement of geldanamycin was performed by using the isocratic HPLC method as previously described [37]. For the determination of geldanamycin, the cell pellet and agar were extracted with ethylacetate.

RESULTS AND DISCUSSION

Cell Growth and Geldanamycin Production of *sha4*-Carrying *S. hygroscopicus* Under Salt Stress

Our strategy, heterologous expression of *sha4* in *S. hygroscopicus* JCM 4427, allowed us to demonstrate its cation specificity on K^+ and its roles for cell growth and geldanamycin production under K^+ stress. Surface-grown culture of the *sha4*-carrying geldanamycin producer (*S. hygroscopicus* pSha4) was carried out to investigate cell growth, geldanamycin production, and cation specificity of *sha4* under salt stress. No significant difference in cell growth was observed between *S. hygroscopicus* pSha4 and *S. hygroscopicus* pKC1139, the control, when there was no salt stress (data not shown). Significant inhibition of cell growth was observed for both strains at 400 mM of Na^+ with no observable difference in the degree of inhibition between them, as shown in Fig. 1B. At 400 mM of K^+, however, the growth of *S. hygroscopicus* pSha4 was much less inhibited by K^+ stress than that of the control (Fig. 1A).

To investigate the effects of *sha4* on geldanamycin production in the presence of K^+ stress, cultures were

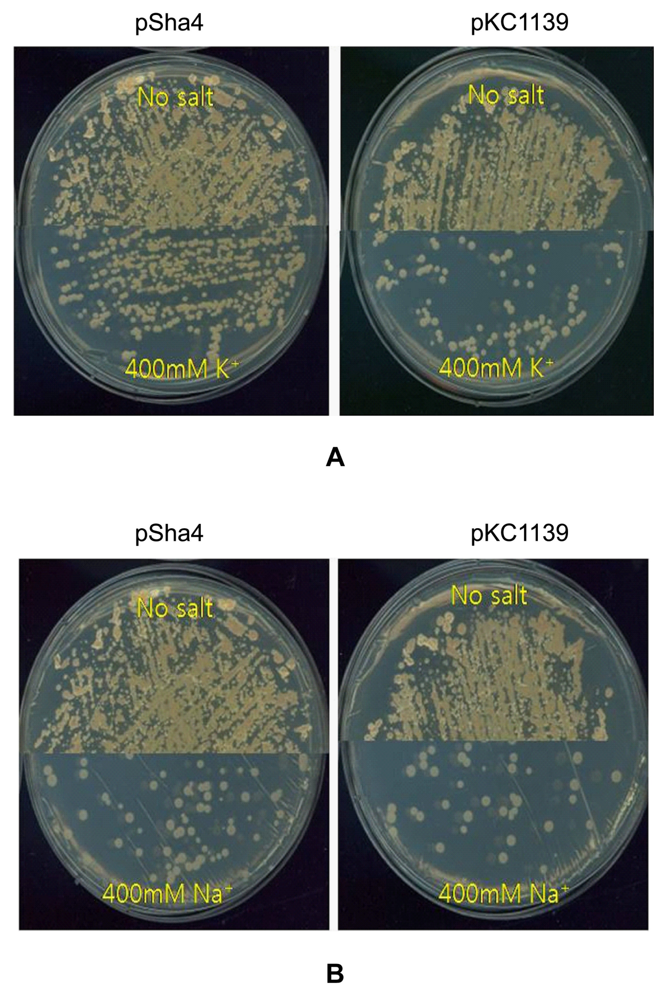

Fig. 1. Cell growth of *sha4*-carrying *S. hygroscopicus* under K^+ and Na^+ stresses.

(**A**) Cell growth of *sha4*-carrying *S. hygroscopicus* under 400 mM KCl stress. (**B**) Cell growth of *sha4*-carrying *S. hygroscopicus* under 400 mM NaCl stress. pKC1139: *S. hygroscopicus* pKC11339. pSha4: *S. hygroscopicus* pSha4.

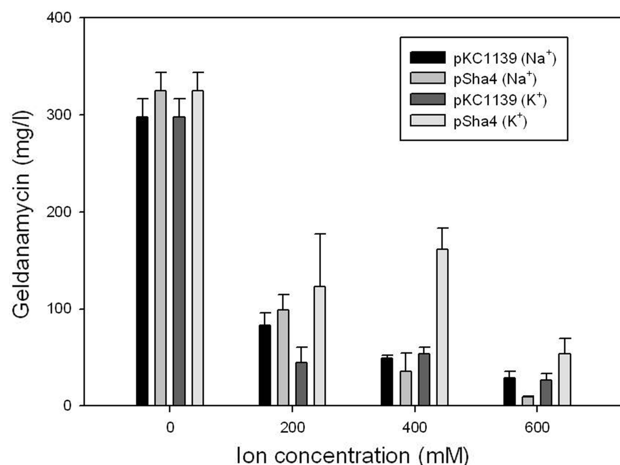

Fig. 2. Extracellular geldanamycin production of *sha4*-carrying *S. hygroscopicus* under K^+ and Na^+ stresses.
pKC1139: *S. hygroscopicus* pKC1139. pSha4: *S. hygroscopicus* pSha4.

carried out at various concentrations of K^+ ranging from 0 to 600 mM. However, in the presence of Na^+ stress, no significant enhancement was observed (Fig. 2). At 200–600 mM of K^+, a significant inhibition of geldanamycin biosynthesis was observed in the cultures of *S. hygroscopicus* pKC1139 than in those of *S. hygroscopicus* pSha4. This result indicated that the expression of *sha4* had enhanced K^+ stress tolerance in geldanamycin biosynthesis. No geldanamycin production was observed in the cultures of both strains over 800 mM of K^+ (data not shown). Interestingly, it was observed that geldanamycin secretion was greatly enhanced with the expression of *sha4* (Fig. 3). In *S. hygroscopicus* pKC1139 culture, only 69% of geldanamycin was secreted, whereas 93% of gelanamycin was secreted in *S. hygroscopicus* pSha4 culture.

The cell growth and extracellular geldanamycin production data given in Fig. 1 and 2 clearly show that the *sha4*-carrying *S. hygroscopicus* had significant effects on K^+ tolerance. Although the *sha4*-carrying geldanamycin producer was susceptible to inhibitions by high K^+ concentration, it showed a higher cell growth and extracellular geldanamycin production than the control at each K^+ concentration, implying that *sha4* affected not only cell growth but also geldanamycin secretion positively (Fig. 2 and 3). However, no significant enhancement of cell growth and geldanamycin production were observed for high Na^+ concentrations, indicating *sha4* has no specificity to Na^+. From this result, we concluded that *sha4* had a K^+ specificity only.

Cell Growth of *sha4*-Carrying *E. coli* under K^+ Stress

The *sha4* transformants of *E. coli* were grown in liquid LBK supplemented with 100 mM of K^+ for growth characterization and the result is presented in Fig. 4. The maximum OD_{600} of *E. coli* K12 transformed with empty pKC1139 (K12C) was 5.7 at 100 mM of K^+, whereas the Δ*kefB* strain transformed with empty pKC1139 (KKC) was 3.3. For the complementation experiment, the pSha4-transformed Δ*kefB* strain (KKS4) was also grown at 100 mM of K^+. K12S4, the pSha4-transformed *E. coli* K12, showed less cell lysis at the stationary phase than its wild type, K12C. A partial restoration of the impaired cell growth of KKC from *kefB* deletion by *sha4* dosage was obvious when we compared the final cell concentrations of KKC and KKS4. In our preliminary study, however, no

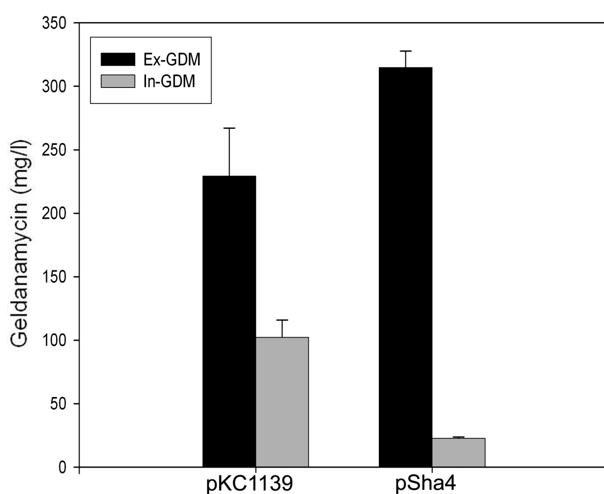

Fig. 3. Geldanamycin secretion of *sha4*-carrying *S. hygroscopicus*.
sha4-carrying *S. hygroscopicus* was grown in geldanamycin production medium containing 10 mM KCl. Extracelluar geldanamycin (Ex-GDM) and intracellular geldanamycin (In-GDM) were investigated. pKC1139; *S. hygroscopicus* pKC1139, pSha4: *S. hygroscopicus* pSha4.

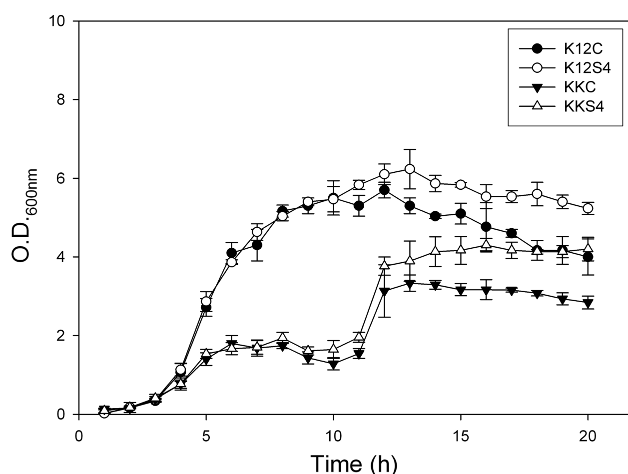

Fig. 4. Cell growth of *sha4*-carrying *E. coli* under salt stress.
K12C, K12S4, KKC, and KKS4 were grown in LBK containing 100 mM KCl. All experiments were carried out at least twice. K12C: *E. coli* K12 pKC1139. K12S4: *E. coli* K12 pSha4. KKC: Δ*kefB E. coli* K12 pKC1139. KKS4: Δ*kefB E. coli* K12 pSha4.

significant *sha4* dosage effect was observed below 100 mM of K^+. The *kefB*-deletion mutants of KKC and KKS4 showed a two-stage growth pattern.

The deletion of *kefB* from *E. coli* (KKC) caused a cell growth inhibition by high K^+ concentration, indicating that this gene provided the host with K^+ stress tolerance. A strain carrying pSha4, KKS4, showed a higher maximum cell concentration than KKC, but a lower value than K12C, demonstrating that the impaired cell growth of KKC from *kefB* deletion was partially restored by *sha4* dosage. In addition, a higher cell growth of K12S4 than that of K12C was observed in the presence of K^+, indicating that *sha4* even fortified K^+ tolerance of the wild-type strain, which had its own K^+/H^+ antiporter system (Fig. 4). From these results, *sha4* was considered to have important roles for cell survival under high K^+ condition, like other K^+or cation/H^+ antiporters [1, 6, 33].

K^+ Efflux Activity of *sha4*

According to a protein BLAST at the NCBI (http://www.nih.gov), *sha4* has homology with many microbial K^+/H^+ or Na^+/H^+ antiporters. Specifically, *sha4* has a high similarity to currently known K^+/H^+ or putative Na^+/H^+ antiporters of *Streptomyces* species, 86% identity with SAV3676 of *S. avermitilis* MA4680, and 81% identity with SGR4293 of *S. griseus* NBRC13350. The gene of *kefB* is for a well-studied K^+ efflux pump of *E. coli*. The lack of K^+ efflux activity of the *kefB*-deficient-strain has already been reported [2]. Although the homology of *kefB* and *sha4* is 26% based on protein BLAST, we hypothesized that *sha4* could restore the K+ efflux activity in the *kefB*-deficient strain because *sha4* is annotated as a putative K^+/H^+ antiporter. To elucidate the K^+ efflux activity of *sha4*, the *sha4*-carrying *E. coli* were grown in the salt-free LB until the stationary phase, and then 400 mM of KCl was added to the cultures. The intracellular potassium concentration, $[K^+]_{intracellular}$, was monitored for 10 min, as shown in Fig. 5. It was found that the *sha4*-carrying K^+ efflux-deficient strain (KKS4) showed much faster K^+ extrusion to return to the normal level than the control (KKC) after the K^+ shock. In the cultures of KKC and KKS4, the intracellular K^+ concentration was increased upto about 330 mM after 1 min of K^+ treatment and then decreased. Only the intracellular K^+ of KKS4, however, decreased rapidly to the original level.

Acidic-pH Shock Tolerance

To investigate the *sha4* effects on acidic-pH shock tolerance, acidic-pH shock was applied to pSha4-carrying *E. coli* cultures. The *sha4* transformants were grown in the salt-free LB upto OD_{600} 0.5, and then two modes of acidic-pH shock were applied for 15 min. The results are shown in Fig. 6. It was found that the *sha4* transformants (K12S4 and KKS4) showed a higher acidic-pH shock tolerance than the controls (K12C and KKC), respectively. Thirty-five percent of K12C and 74% of the K12S4 survived at pH 4.5, and 24% of K12C and 52% of K12S4 survived at pH 3.5. The *E. coli* strain deficient in K^+/H^+ antiporter (KKC) showed high acidic-pH shock susceptibility. In the case of KKC, cell viability was below 15% after an acidic-pH shock dropped down to pH 4.5, indicating the deletion of *kefB* increased susceptibility to acidic-pH shock. KKS4 showed an enhanced tolerance to the acidic-pH shock than KKC, suggesting *sha4* dosage partially had restored acidic-pH shock tolerance. Overall, the *sha4* gene conferred about 2-fold increased acidic-pH shock tolerance upon its host when transformed into wild-type *E. coli* K12 or its K^+/H^+ antiporter-deficient strains.

In this work, the deletion of *kefB* increased acid sensitivity, suggesting this gene is related to acidic-pH shock resistance. The heterologous expression of *sha4* in all of the *E. coli* strains tested in this study enhanced cell

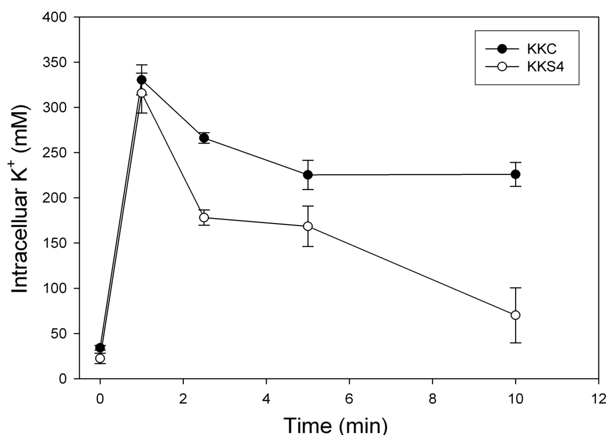

Fig. 5. Cation efflux activity of *sha4*-carrying *E. coli*.
Intracelluar $[K^+]$ profile of KKC and KKS4 after 400 mM KCl shock. KKC: *ΔkefB E. coli* K12 pKC1139. KKS4: *ΔkefB E. coli* K12 pSha4.

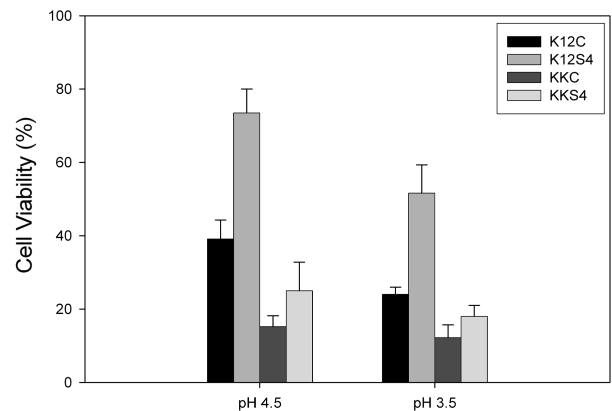

Fig. 6. Acidic-pH shock tolerance of *sha4*-carrying *E. coli*.
Cell viability was determined by CFU. K12C: *E. coli* K12 pKC1139. K12S4: *E. coli* K12 pSha4. KKC: *ΔkefB E. coli* K12 pKC1139. KKS4: *ΔkefB E. coli* K12 pSha4.

viability under acidic-pH shock condition. When *sha4* was expressed in the *E. coli* mutant with *kefB* deleted in the complementation experiment, it was observed that *sha4* partially restored the impaired acidic-pH shock tolerance of the *E. coli* mutant (Fig. 6). This indicated that *kefB*, the K^+/H^+ antiporter of *E. coli*, is essential for cell survival under a condition of abrupt pH change, and the *sha4* had an ability to, at least partially, take over the roles of *kefB*.

An acidic-pH shock was also applied to surface-grown cultures of *sha4*-carrying *S. hygroscopicus* and wild-type *S. hygroscopicus* JCM4427. Cell growth inhibition was observed for both strains with an acidic-pH shock down to pH 4.0. No significant *sha4* dosage effects on acidic-pH shock tolerance were observed when it was overexpressed in the wild-type *S. hygroscopicus*, contrary to the earlier-mentioned case of *sha4*-carrying *E. coli*, which showed an improved pH shock tolerance (Fig. 6). Whereas other currently known cation/H^+ antiporters had tolerance to alkaline condition [27, 32], *sha4,* being expressed in *E. coli*, enhanced tolerance to the acidic-pH shock condition.

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