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Mutation of His-157 in the Second Pore Loop Drastically Reduces the Activity of the *Synechocystis* Ktr-Type Transporter▽

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Mutation of a conserved His-157 in the second pore loop of KtrB drastically reduced the activity of the K^+ transporter from *Synechocystis* sp. strain PCC 6803. This result suggests that His-157 plays an essential role in the K^+ transport activity of the transporter system.

The Ktr/Trk/HKT superfamily of K^+ transporters, which is likely to have evolved from two membrane-spanning K^+ channels, has a fourfold repeated membrane-pore-membrane motif (Fig. 1A) (2, 6, 8). KtrB is the K^+-translocating subunit of the Ktr system from *Synechocystis* sp. strain PCC 6803 (10) and *Vibrio alginolyticus* (10). The Ktr system mediates K^+ uptake in *Synechocystis* sp. strain PCC 6803. It consists of three kinds of subunits, the transmembrane KtrB subunit and the peripheral KtrA and KtrE subunits (9). Previous studies have shown that the Ktr system is essential for the adaptation of *Synechocystis* sp. strain PCC 6803 to salinity stress and high osmolality, and in its transport activity is dependent on the proton motive force (9). *Synechocystis* KtrB possesses two histidines, one located at the extracellular and the other in the intracellular space. The extracellular His-157 in the second pore loop (P_B) is well conserved among Ktr-related proteins of prokaryotic origin (Fig. 1B). In plant K^+ channels from *Solanum tuberosum* (KST1) and *Arabidopsis thaliana* (AKT3), the histidines located at the extracellular space were identified as a determinant for pH dependency (4, 5). The presence of the conserved extracellular His prompted us to investigate the role of His-157 in the activity of *Synechocystis* Ktr.

First, we generated three His variants (H157A, H157E, and H157K) by the overlap extension PCR technique (11). In addition, since another positive residue, Arg-149, is present in P_B, an R149E variant was constructed to compare the transport activities of the His variants. The PCR products were inserted into pPAB404, and mutations were confirmed by DNA sequencing. Native and variant forms were expressed in *Escherichia coli* LB2003 harboring *ktrA* and *ktrE*. *E. coli* LB2003, which lacks all three K^+ uptake systems, was used to verify the K^+ transport activity of KtrB (14).

We first examined the effect of the histidine mutation on the activity of KtrB by performing a complementation test and a K^+ uptake assay to determine the kinetic parameters. The K^+ uptake assay experiment was conducted by the silicone oil filtration technique, and the K^+ content of the cell pellets was determined by flame photometry (13). In medium supplemented with 7.5 mM KCl, the R149E variant showed better growth than the three His variants did (Fig. 2A). In control experiments, the growth test was carried out using the medium containing K_2SO_4 instead of KCl (Fig. 2A). The growth profile was consistent with that for growth in the medium containing KCl. The control experiments indicate that the depressed growth of the His variants at 7.5 mM KCl was due to K^+ deprivation but not due to Cl^-. The initial net K^+ uptake of

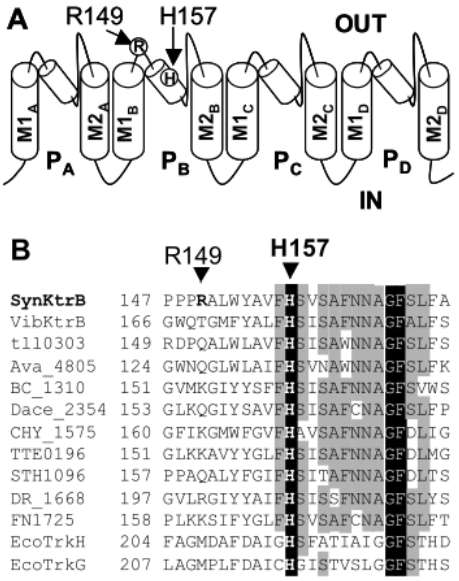

FIG. 1. Alignment and position of His-157 in the P_B of the KtrB subunit of the *Synechocystis* KtrABE system. (A) Predicted topology of *Synechocystis* KtrB. His-157 and Arg-149 are located in P_B. (B) Amino acid sequence alignment of P_B of KtrB and KtrB-related proteins. SynKtrB, *Synechocystis* sp. strain PCC 6803 KtrB (10); VibKtrB, *Vibrio alginolyticus* KtrB (11); EcoTrkH, *Escherichia coli* TrkH (12); EcoTrkG, *Escherichia coli* TrkG (12). Other KtrB-related proteins found in the databases (by use of NCBI BLAST) that have not been functionally characterized yet are the following: tll0303, *Thermosynechococcus elongatus* BP-1; Ava_4805, *Anabaena variabilis* ATCC 29413; BC_1310, *Bacillus cereus* ATCC 14579; Dace_2354, *Desulfuromonas acetoxidans* DSM 684; CHY_1575, *Carboxydothermus hydrogenoformans* Z-129; TTE0196, *Thermoanaerobacter tengcongensis* MB4; STH1096, *Symbiobacterium thermophilum* IAM14863; DR_1668, *Deinococcus radiodurans* R1; FN1725, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586.

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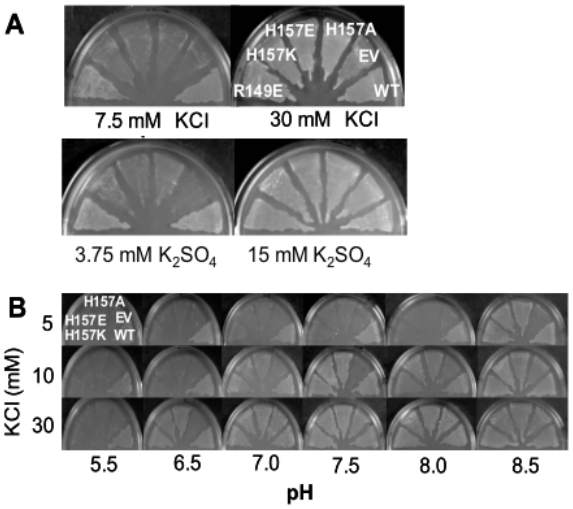

FIG. 2. Growth test of *E. coli* LB 2003. (A) Growth test of *E. coli* LB 2003 harboring WT KtrB or H157A, H157E, H157K, or R149E variants on synthetic solid medium containing 7.5 mM or 30 mM KCl, 10 mM HEPES-NaOH (pH 7.5). Control experiments using K_2SO_4 (3.75 and 15 mM) are shown. (B) Growth test of *E. coli* LB 2003 harboring WT or His variants at various pH values on synthetic solid medium containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH (pH 5.5 and 6.5), 10 mM HEPES-NaOH (pH 7.0, 7.5, and 8.0), or 10 mM Tricine-NaOH (pH 8.5) at different KCl concentrations. Photographs were taken after overnight incubation at 30°C.

the His variants was significantly lower, i.e., more than 10-fold and more than 5-fold lower than that for the wild type (WT) and that for the R149E variant, respectively (Table 1). The V_{max} value data indicated that replacement of His-157 by Ala, Glu, or Lys resulted in a significant reduction in the K^+ uptake activity of the variants (Table 2). The apparent K_m values for K^+ for the three His variants at pH 7.5 exhibited an increase by a factor of at least 24-fold compared to the wild type. The kinetic parameters among the His variants at pH 7.5 were not significantly different (Table 2). These results show that His-157 had a role more crucial than that of Arg-149 and was irreplaceable at that position by the other residues to restore the optimum activity seen in the wild type.

Next, we determined the effect of the histidine mutation on K^+ uptake activity at various pH values through complementation testing and determination of kinetic parameters. The

TABLE 1. Initial velocities of K^+ uptake

Strain or characteristic	Initial velocity of K^+ uptake (nmol · min^{-1} · mg^{-1} dw)[a]
WT	96 ± 7
H157A	6.1 ± 1
H157E	8.6 ± 1
H157K	4.2 ± 0.5
R149E	48 ± 4

[a] The assay was done in HEPES-NaOH buffer (pH 7.5) containing 94 mM NaCl. dw, dry weight. Cells were taken from the cell suspension after a 1-min addition of 1 mM KCl. Values are averages of results from three independent experiments ± standard deviations.

TABLE 2. Kinetic parameters for the K^+ uptake by *E. coli* LB2003 containing WT or variant KtrB

KtrB variant	Kinetic parameter[a]											
	Apparent K_m for K^+ (mM) at pH:						V_{max} (nmol · min^{-1} · mg^{-1} dw^b) at pH:					
	5.5	6.5	7.0	7.5	8.0	8.5	5.5	6.5	7.0	7.5	8.0	8.5
WT	0.26 ± 0.5*	0.18 ± 0.3*	12 ± 0.3*	0.07 ± 0.009 0.5 ± 0.1	0.09 ± 0.02**	0.11 ± 0.02*	81 ± 6	104 ± 7	107 ± 8	116 ± 9 70 ± 5	102 ± 11	101 ± 11
R149E												
H1571	ND	ND	2.3 ± 0.2**	2.1 ± 0.2	2.7 ± 0.4**	ND	ND	ND	17 ± 3	20 ± 3	21 ± 4	ND
H157E	ND	ND	2.6 ± 0.4**	1.8 ±0.4	3.1 ± 0.6**	ND	ND	ND	26 ± 4	25 ± 4	21 ± 3	ND
H157K	ND	ND	2.7 ± 0.7**	1.7 ± 0.5	2.4 ±0.5**	ND	ND	ND	13 ± 2	11 ± 2	11 ± 2	ND

[a] Kinetic parameters were obtained by use of Eadie-Hofstee plots of the initial rates of K^+ uptake. K^+ uptake assays were performed in 200 mM MES-NaOH (pH 5.5 and 6.5), 200 mM HEPES-NaOH (pH 7.0, 7.5, and 8.0), and 200 mM Tricine-NaOH (pH 8.5). The concentration of added KCl was varied between 0.05 and 2.0 mM (for WT), between 0.1 and 3 mM (for R149E), and between 0.5 and 6.0 mM (for His variants). The values are averages for three independent experiments ± standard deviations. Student's *t* test was used to compare the apparent K_m values obtained at various pH values to that obtained at pH 7.5 (control). *, significant ($P = 0.05$); **, not significant. ND, not determined.

[b] dw, dry weight.

functional complementation test using the *E. coli* LB2003 mutant showed that the wild type grew well at all pH values of the medium, indicating that the Ktr system from *Synechocystis* sp. strain PCC 6803 could operate in a wide range of pH values when expressed in *E. coli.* Growth test data for liquid culture also supported this result (data not shown). His variants could not rescue the mutation of the *E. coli* mutant LB2003 on acidic medium (Fig. 2B). The lack of complementation by His variants might be due to its low K^+ uptake activity, which can lead to severe inhibition of growth. *Escherichia coli* cannot maintain an internal pH of more than 2 units higher than the external pH (3). Under acidic growth conditions, cytoplasmic accumulation of K^+ and proton extrusion are involved in cytoplasmic pH regulation (1, 7). This result showed the physiological role of the K^+ uptake system in the acidic pH condition. Thus, in this respect, the accumulation of K^+ through the high activity of wild-type KtrB supported the hypothesis for the regulation of internal pH homeostasis for normal growth. The lower activity of His variants, which did not compensate for this, led to the cessation of metabolic pathways and cell growth in medium with a lower pH (Fig. 2B).

Changes in the kinetic parameters of the wild type at various pH values were observed. Decreases in affinity to K^+ and V_{max} were observed when the external pH of the buffer used for the uptake assay was decreased or increased from the optimum pH (pH 7.5) (Table 2). A marked decrease in V_{max} was observed at pH 5.5 (Table 2). The kinetic parameter results for WT at various pH values showed that the activity of the transporter is pH dependent. To test whether His-157 is involved in the pH response, we determined the kinetic parameters of the His variants at different pH values. The apparent K_m and V_{max} values could be obtained only at pH 7.0 and 8.0 from the three His variants, and the values did not show significant difference from those obtained at pH 7.5 (Table 2). The involvement of His-157 in the pH response of transporter KtrB was not found.

Atomic-scale models of Ktr transporters proposed by Durell and Guy have shown that His-157, together with Phe-156 and Ser-158, faced the transport pathway (2). The three residues are likely to be placed in juxtaposition to Gly-166, which is a constituent of the selectivity filter in P_B (2, 8). The replacement of His-157 by Ala, Glu, or Lys might affect the proper conformation required for optimal activity.

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