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Kup is the major K^+ uptake system in *Escherichia coli* upon hyper-osmotic stress at a low pH

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Abstract The K^+ uptake was observed in washed cells of *Escherichia coli*, wild-type, upon hyper-osmotic stress at pH 5.5 when glucose was supplemented. This uptake had apparent a K_m of 0.58 mM and V_{max} of 0.10 μmol K^+/min/mg protein. Such a K^+ uptake was investigated using a mutant defective in Kdp and TrkA but with a functional Kup and a mutant defective in Kdp and Kup but having an active TrkA. The K^+ uptake to reach the steady state level as well as the initial K^+ influx rate in the first mutant were at least 3.5-fold greater than these values with the second mutant and similar to those of the wild-type. Such differences in the K^+ uptake activity were correlated with K^+ requirements for growth of these mutants. Moreover, the K^+ uptake in the wild-type was blocked by a protonophore (carbonyl cyanide *m*-chlorophenylhydrazone). Valinomycin, arsenate and *N,N′*-dicyclohexylcarbodiimide were not effective in changing the K^+ uptake. It is suggested that Kup is the major K^+ uptake system in *E. coli* upon hyper-osmotic stress at a low pH.

Key words: K^+ uptake; Kup; TrkA; Low pH; *Escherichia coli*

1. Introduction

K^+ uptake systems are more extensively studied in *Escherichia coli*, where TrkA and Kup (formerly TrkD) form a family of constitutive low affinity transport systems (for a review, see [1]). Upon hyper-osmotic stress at neutral and slightly alkaline pH, TrkA is the major system with a relatively high rate of K^+ uptake [2–4]. This system is assumed to be a sum of two mechanisms, TrkH composed of the *trkA*, *trkE* and *trkH* gene products and TrkG composed of the *trkA*, *trkE* and *trkG* gene products. Only the *trkA* gene product is absolutely needed for normal activity of both mechanisms [4]. One of the features of TrkA is in requiring both ATP and a transmembrane proton electrochemical gradient $\Delta\mu_H^+$ [3,5,6], however this system does not belong to either of the two common groups of active transport systems, primary ATP-powered pumps or secondary $\Delta\mu_H^+$-driven porters. In aerobically grown bacteria, TrkA is likely to operate as a secondary porter using $\Delta\mu_H^+$ to drive the K^+ uptake and ATP activates its function [7]. But in fermenting cells grown under anaerobic conditions, TrkA requires the F_0F_1 ATP synthase [8] and a relationship between these two systems and a different mode of TrkA operating as an ATP-powered pump are proposed [3,8–11]. Genetic evidence on the separate existence of Kup encoded by the single *trkD* gene has been provided recently [4]. This system with a low rate of K^+ uptake [2,4] is assumed to modestly contribute to increase in K^+ uptake at low concentrations of this ion. It becomes important when the activity of TrkA is insufficient and Kdp, encoded by the *kdpABC* operon and known to be the K^+ uptake system with a high affinity [2], is not induced. However, Kup seems not to be involved in the osmotic regulation of bacteria at hyper-osmotic stress [7,12]. Unlike TrkA, this system operates as a secondary porter using only $\Delta\mu_H^+$ [5,7]. Such a mode of Kup is probable since the nucleotide sequence of a relevant gene shows no sign of an ATP-binding motif [13]. The operation of K^+ uptake systems under each set of conditions has to be clarified. It may be a rather important way to understand why bacteria have multiple K^+ transport systems.

E. coli is able to grow at a low pH, for instance below 6.0 [14–16], when the cytoplasmic pH is lowered and transport systems might be changed. It has been shown that the rate of K^+ uptake through TrkA under aerobic conditions is strongly decreased with lowering the external and cytoplasmic pH by a weak acid [17]. However, weak acids do not affect the intracellular K^+ level [18]. At the same time, at a low pH, these bacteria are supposed to have an increased $[K^+]_{in}$ when fermentation acids are present. K^+ provides a counteraction for fermentation acid and allows bacteria to tolerate even greater amounts of fermentation anions (for review see [19]). Thus, at a low pH, a high K^+ level in *E. coli* may be maintained by a system different from TrkA and Kup may therefore become important.

The results of the present study show that Kup is responsible for the majority of the K^+ uptake by *E. coli* upon hyper-osmotic stress at pH 5.5.

2. Materials and methods

2.1. Bacterial strains and growth

E. coli wild-type strain W3110 and mutants defective in different K^+ uptake systems, TK1001 (Δ*kdpABC5 trkD1*) [2], TK1110 (Δ*kdpABC5 trkA405*) [20], and in the F_0F_1 ATP synthase, KF11 (*atpD11* (formerly *uncD11*) [21], were used throughout. All strains derived from *E. coli* K12 were also F^- λ^-. W3110 was from Dr Y. Anraku (Tokyo University, Tokyo, Japan), TK1001 and TK1110 were generously supplied by Dr W. Epstein (The University of Chi-

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Abbreviations: F_0F_1, the H^+-translocating ATP synthase; TrkA(G,H), Kup, the constitutive low affinity K^+ uptake systems in *E. coli*; Kdp, the inducible high affinity K^+ uptake system in *E. coli*; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N′*-dicyclohexylcarbodiimide; EDTA, ethylenediaminotetraacetic acid; $\Delta\mu_{H^+}$, transmembrane proton electrochemical gradient; $\Delta\Psi$, the membrane electric potential

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cago, Chicago, IL, USA) and KF11 was a gift from Dr M.Futai (Osaka University, Osaka, Japan).

Bacteria were grown at 37°C, either anaerobically in stationary tubes filled with growth medium or aerobically in vigorously shaken tubes partially filled with growth medium such that the ratio of medium volume to tube volume was 1:5. Fermentation or aerobic respiration in bacteria under these conditions was described previously [11]. The growth medium consisted of 20 g of polypepton, 5 g of NaCl, 2 g of K_2HPO_4 and 2 g of glucose/l. The medium pH was adjusted to 5.5 or 7.3 by the addition of HCl or NaOH. Bacterial growth was monitored measuring the absorbance of the culture at 600 nm using a colorimeter.

2.2. Study of the K^+ uptake

The K^+ uptake by bacteria was investigated by measuring the intracellular K^+ level ($[K^+]_{in}$) and external K^+ activity ($[K^+]_{ex}$). $[K^+]_{in}$ was determined using the atomic absorption spectrophotometer (Hitachi Z-8000, Japan) as described previously [22], except that samples were centrifuged through lauryl bromide [23]. $[K^+]_{ex}$ was defined with a glass selective electrode as described elsewhere [3,8,9,11]. To study the K^+ uptake, cells were grown to the mid-logarithmic phase and harvested at an absorbance of about 0.4 when the medium pH was decreased to 5.2 and 6.9 from an initial pH of 5.5 and 7.3, respectively. Then, cells were washed and transferred into a chamber with 250 mM Tris-phosphate, pH 5.5 when the growth pH was 5.5, or pH 7.5 when the growth pH was 7.3, consisting of 0.4 mM $MgSO_4$, 1 (or as indicated) mM KCl and 1 mM NaCl (solution A). Glucose, 22 mM, was subsequently supplemented. Bacteria were subjected to hyper-osmotic stress by transfer from distilled water or 20 mM EDTA into solution A with a high osmolarity, whereas transfer of cells from 0.8 M sucrose into solution A is hypo-osmotic stress. It should be noted that during washing with distilled water or EDTA, cells partially became K^+ depleted [10,17,23] and, therefore, suitable for the K^+ uptake study. Moreover, EDTA treatment made the bacterial cytoplasmic membrane accessible to hydrophobic reagents. To determine a small change in $[K^+]_{ex}$, cells were concentrated into a chamber at a density of about 5×10^{10}–10^{11} cells/ml. Capped chambers and chambers with pumping of oxygen were used for anaerobically and aerobically grown bacteria, respectively. All assays were done at 37°C. The apparent K_m and V_{max} for the K^+ uptake by bacteria were determined with a Lineweaver-Burk plot of the initial K^+ influx. When used, cells were incubated with 10 mM arsenate or 0.2 mM DCCD for 10 min prior to the addition of glucose.

2.3. Determination of the K^+ requirement for growth

The K^+ requirement for growth of bacteria was determined as described [10]. The growth K_m was defined as the K^+ concentration required to achieve half the maximal growth rate in media containing high K^+ concentrations.

2.4. Others and chemicals

Protein was determined as described in Lowry et al. [24] using bovine serum albumin as a standard. The bacterial count of diluted suspension was estimated by cell absorbance and a calibrating curve relating the absorbance to the number of colonies grown on solid media. Polypepton (Kyokuto, Japan), CCCP, DCCD, valinomycin (Sigma, USA) and other chemical reagents were of analytical grade.

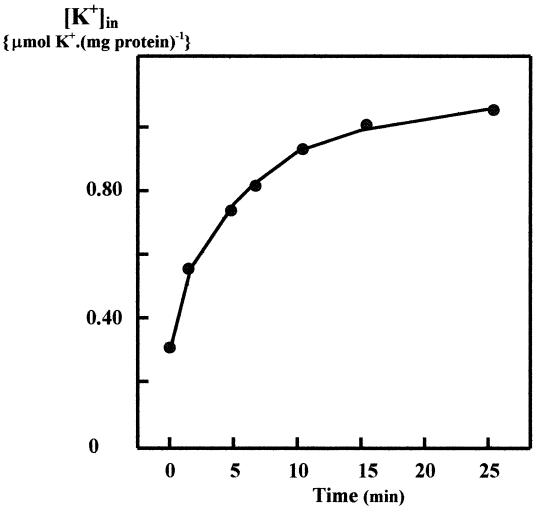

Fig. 1. Kinetics of K^+ accumulation by the *E. coli* wild-type strain W3110 upon hyper-osmotic stress at pH 5.5. Cells grown under anaerobic conditions at initial pH 5.5 were washed with distilled water and subjected to hyper-osmotic stress as described in Section 2. Glucose, 22 mM, was supplemented at time zero. Average data of $[K^+]_{in}$ are represented, S.D. do not exceed 5%.

3. Results and discussion

3.1. The K^+ uptake by bacteria upon hyper-osmotic stress at a low pH

The K^+ level of *E. coli* wild-type strain W3110, grown under anaerobic conditions at an initial pH 5.5 and washed with distilled water, was determined to be 0.30 ± 0.01 μmol K^+/mg protein, which was less than those of cells washed with 20 mM EDTA (0.42 ± 0.01 μmol K^+/mg protein) or 0.8 M sucrose (0.67 ± 0.02 μmol K^+/mg protein). The K^+ uptake was shown upon hyper-osmotic stress at pH 5.5 in the cells washed with distilled water (Fig. 1) or EDTA (Table 1) when glucose was supplemented. To reach the steady state level, K^+ accumulation was actuated over 10 min (Fig. 1). An increase of $[K^+]_{in}$ was not observed either in the absence of glucose (Table 1) or upon hypo-osmotic stress (not shown). These data suggest that the K^+ uptake could be carried out through some transport systems in response to hyper-osmotic stress. Kdp was noted to be induced when the external K^+ activity is less than 0.02 mM [2] and it was therefore not switched on under our growth conditions. There is also an unidentified system in *E. coli*, TrkF, with a very low affinity and a low rate of K^+ uptake [1–3,8,25]. It could contribute

Table 1
K^+ uptake by the *E. coli* wild-type strain W3110 upon hyper-osmotic stress at pH 5.5

Ionophores	Glucose (22 mM)	$[K^+]_{in}$ (μmol K^+/mg protein) at 7 min	Change in $[K^+]_{in}$ (% of the value at time zero)
No additions	−	0.42 ± 0.01	100
	+	0.75 ± 0.02	179
CCCP (0.05 mM)	+	0.44 ± 0.03	105
Valinomycin (0.01 mM)	+	0.79 ± 0.03	188
CCCP (0.05 mM) and valinomycin (0.01 mM)	+	0.22 ± 0.01	52

Effects of ionophores on the K^+ uptake.
Cells grown under anaerobic conditions at initial pH 5.5 were treated with 20 mM EDTA for 10 min and subjected to hyper-osmotic stress (see Section 2).
At time zero when glucose was supplemented (indicated) and at 7 min, K^+ was measured as described in Section 2.
The $[K^+]_{in}$ at time zero was 0.42 ± 0.01 μmol K^+/mg protein.
Avarage data are represented with S.D.

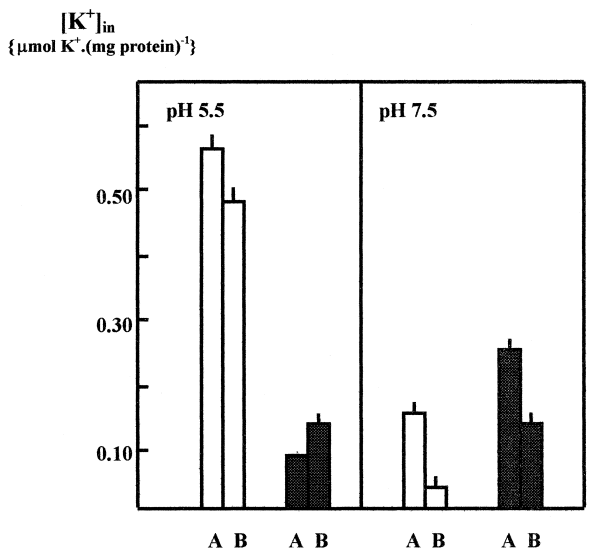

Fig. 2. The K^+ uptake to reach the steady state level by *E. coli* mutants defective in different K^+ uptake systems. The mutants TK1110 deleted for Kdp and defective in TrkA but having a functional Kup (non-filled rectangles) and TK1001 deleted for Kdp and defective in Kup but having an active TrkA (filled rectangles) were grown under anaerobic (A) and aerobic (B) conditions at pH 5.5 or pH 7.3 as shown. Cells were then washed with distilled water and transferred into solution A with pH 5.5 or pH 7.5, respectively (indicated), as described in Section 2. In others see the legends to Fig. 1 and Table 1.

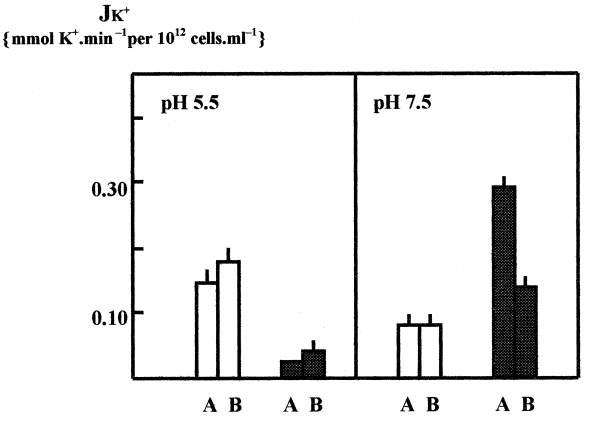

Fig. 3. K^+ influx rates in *E. coli* mutants defective in different K^+ uptake systems. The initial K^+ influx rate (J_K^+) was determined using a selective glass electrode as described in Section 2. In others see the legends to Fig. 2.

little in the K^+ uptake at a low external activity of the ion we used. Therefore, TrkA and Kup may mediate the K^+ uptake under our conditions at a low pH. It is less plausible that Kup is the major system upon hyper-osmotic stress, since at alkaline pH, Kup is not supposed to be active in hyper-osmotic media [7,12]. The role of Kup seems to be ignored since at alkaline pH this system makes a small contribution compared to TrkA.

Kinetic parameters of the K^+ uptake determined show that K_m is equal to 0.58 mM, which was reported for Kup [2,4] and (in certain conditions) for TrkA [17] at neutral and slightly alkaline pH, while the V_{max} of 0.10 μmol K^+/min/mg protein is close to that of Kup at a neutral and slightly alkaline pH [2,4] and different from the value documented with TrkA at pH 6.15 [17]. Since different values of K_m were established with Kup and TrkA [2–4,17] and the V_{max} could be acutely changed with the external pH [17], the kinetic study is not at all convincing to draw the conclusion which system is responsible for the majority of the K^+ uptake upon hyper-osmotic stress at a low pH.

3.2. On the nature of K^+ uptake

In order to establish the nature of K^+ uptake activity upon hyper-osmotic stress in *E. coli* grown at a low pH, the K^+ uptake was studied in the mutants TK1001 and TK1110 having a functional TrkA and Kup, respectively. The $[K^+]_{in}$ of washed cells were 0.15 ± 0.01 and 0.24 ± 0.02 μmol K^+/mg protein, respectively, in the mutants TK1001 and TK1110 grown under anaerobic conditions and these values were lower than that in the wild-type. The K^+ uptake upon hyper-osmotic stress at pH 5.5 to nearly reach the steady state level by the mutant TK1110 grown under either anaerobic or aerobic conditions was at least 3.5-fold greater than that by TK1001 (Fig. 2). These data allow us to suggest that Kup rather than TrkA is responsible for the majority of the K^+ uptake at a low pH under our conditions. In contrast to a low pH, under either anaerobic or aerobic growth, the K^+ uptake activity upon hyper-osmotic stress at pH 7.5 in the mutant TK1110 was much lower than that in TK1001 (Fig. 2). The latest results confirm the observation that TrkA is the major K^+ uptake system at neutral and slightly alkaline pH [2–4,8,11,12]. Moreover, the rates of the initial K^+ influx by the mutant TK1110 under anaerobic and aerobic growth at pH 5.5 were respectively 6- and 4-fold higher than the rates of TK1001. The rate of the K^+ influx by the mutant TK1110 at pH 7.5 was lower than that by TK1001 (Fig. 3). Such a differ-

Table 2
K^+ requirements for growth of *E. coli* mutants defective in different K^+ uptake systems

Strain	Relevant genotype	Growth conditions	Growth K_m (mM) pH 5.5	pH 7.3
TK1110	*ΔkdpABC5 trkA405*	Anaerobic	0.10	0.35
		Aerobic	0.05	0.30
TK1001	*ΔkdpABC5 trkD1*	Anaerobic	0.58	0.14
		Aerobic	0.30	0.08

Cells in the mid-logarithmic phase of growth were washed with media without K^+, pH 5.5 or pH 7.3, and then inoculated in series of tubes containing saline media of different K^+ concentrations and 11 mM glucose, pH 5.5 or pH 7.3, respectively, at a density of approximately 5×10^7–10^8 cells/ml.
The growth rate was determined over the interval where the logarithm of absorbance increased linearly with time and is expressed as 0.693/doubling time.
The growth rates of the mutants at 105 mM K^+ were 0.42 ± 0.02 and 0.80 ± 0.02/h in anaerobic and aerobic conditions, respectively.
Average data from two independent measurements were employed to determine growth K_m (see Section 2).

Table 3
The effect of energy transfer inhibitors on the K^+ uptake by the *E. coli* wild-type and mutants defective in different K^+ uptake systems upon hyper-osmotic stress at pH 5.5

Inhibitor	Change in $[K^+]_{in}$ (% of the value at time zero)		
	W3110 (wild-type)	TK1110 (*kdpABC trkA*)	TK1001 (*kdpABC trkD*)
Arsenate			
20 mM	263	366	103
50 mM	213	300	100
DCCD			
0.2 mM	211	323	80
0.5 mM	215	317	68

Cells washed with distilled water were treated with arsenate, 10 mM, or DCCD, 0.2 mM, for 10 min (see Section 2).
Data are the averages of two independent measurements.
In others see the legends to Table 1.

ence between K^+ influxes by TrkA and Kup at slightly alkaline pH is in accordance with the values reported by Epstein and coworkers [2,4] and Dinnbier et al. [12].

The results of the K^+ uptake activity are correlated with data on K^+ requirements for growth of the mutants TK1001 and TK1110. At pH 5.5, growth of the mutant TK1110 was seen to have a lower K^+ requirement than TK1001, such a requirement was oppositely changed when cells were grown at pH 7.3 (Table 2). The finding with the mutant TK1110 appears to contradict that of Rhoads et al. [2], although the data with TK1001 (Table 2) confirm their result [2]. They reported that under aerobic conditions at pH 5.8, the growth of a mutant defective in Kdp and TrkA had a much higher K^+ requirement than TK1001 and, moreover, the K_m for growth of that mutant lowered at pH 7.0 [2]. This disagreement was probably due to the difference in the medium osmolarity. It might also be possible that the contradiction is due to a different genetic background of mutants. The *trkA* allele in TK1110 was A405, whereas that in TK133 used by Rhoads et al. [2] was A133 and so these mutants were constructed in somewhat different ways. Interestingly, indistinguishable K^+ uptake kinetics but quite different K^+ requirements for growth have been further reported by Dosch et al. [4] with the other mutants having a different *trkA* allele.

3.3. The effect of ionophores and inhibitors on the K^+ uptake

To understand the mode of operation of the K^+ uptake system upon hyper-osmotic stress at a low pH, effects of ionophores and energy transfer inhibitors on the K^+ uptake were further studied. At pH 5.5, the K^+ uptake by the *E. coli* wild-type was found to disappear in the presence of a protonophore (CCCP) (Table 1). The latter might lower $\Delta\mu_H^+$, however precise data on this matter are not yet available. K^+ uptake by the wild-type and the mutant TK1110 was not inhibited by arsenate that affected the K^+ uptake by TK1001 (Table 3). These results together might indicate that a low pH Kup could depend on the $\Delta\mu_H^+$ and not require ATP, although TrkA requires both $\Delta\mu_H^+$ and ATP. Such features of Kup and TrkA have been already established at neutral and slightly alkaline pH [3,5–7]. Moreover, valinomycin was not effective in changing the K^+ uptake by the wild-type, however it decreased $[K^+]_{in}$ in the presence of CCCP (Table 1) and increased the K^+ efflux from cells in media without glucose (not shown). This effect of valinomycin could be explained by the $\Delta\mu_H^+$-dependent operation of the K^+ uptake system in the wild-type. These findings (Tables 1 and 3) are consistent with Kup being the major K^+ uptake system in *E. coli* upon hyper-osmotic stress at a low pH.

In order to study a relation of the K^+ uptake with the F_0F_1 ATP synthase, capable to generate $\Delta\mu_H^+$ under fermentative conditions, effects of DCCD as well as of an *atp* mutation on the K^+ uptake were investigated. Under anaerobic conditions at pH 5.5, DCCD, even at elevated concentration, was unable to inhibit the K^+ uptake by the *E. coli* wild-type or the mutant TK1110 (Table 3), while at alkaline pH this reagent has been previously shown to obliterate the K^+ uptake [3,8,9,11]. Then, in the *atpD* mutant KF11 grown at pH 5.5, the $[K^+]_{in}$ of 0.13 ± 0.01 μmol K^+/mg protein was smaller than that of the wild-type. But at pH 5.5, the K^+ uptake up to the intracellular level of 0.29 ± 0.03 μmol K^+/mg protein was observed upon hyper-osmotic stress in the presence of glucose. Interestingly, such an uptake was absent in the mutant grown at pH 7.3 (not shown), confirming the previous finding [8]. These results show that under anaerobic conditions at a low pH, the K^+ uptake by Kup does not require the functioning F_0F_1. It should be noted that under such conditions at a low pH $\Delta\mu_H^+$ in *E. coli* can be generated by end product extruding and other mechanisms different from the F_0F_1 ATP synthase (for reviews see [1,26]). Moreover, the findings that the K^+ uptake by TrkA was inhibited with DCCD in the mutant TK1001 grown at pH 5.5 (Table 3) as well as it was absent in the *atpD* mutant grown at pH 7.3 (not shown) may support an idea of close relationship of TrkA with F_0F_1. Such a proposal has been advanced for *E. coli* under fermentative growth at slightly alkaline pH [3,8–11,25]. A direct effect of DCCD on the K^+ uptake was ruled out because the reagent did not inhibit the accumulation of this ion by the mutant TK1001 grown under aerobic conditions (not shown).

4. Concluding remarks

E. coli has been shown to uptake K^+ upon hyper-osmotic stress at pH 5.5 (Table 1). At the moderate K^+ activity we used, the K^+ uptake can be mediated by the low affinity TrkA and Kup systems, but at a low pH, the latter is the major system (Figs. 2 and 3). It may be possible to postulate that *E. coli*, having multiple systems for K^+ uptake, uses distinct systems at different pH. TrkA mainly functions at an alkaline pH [2–4,8,11,12] and Kup becomes a more important system at a low pH (the present study). Such an idea seems to be similar to the recent proposal of Sakuma et al. [27] for the sodium ion extrusion systems of *E. coli*, when distinct transporters are operating at different pH values.

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