K⁺ transport in *Vibrio alginolyticus*: isolation of a mutant defective in an inducible K⁺ transport system

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When grown in a synthetic medium containing more than 3 mM K⁺, the marine bacterium *Vibrio alginolyticus* exhibited a K⁺ transport system with apparent K_m and maximum velocity (V_{max}) of 3.0 mM and 1.5 $\mu\text{mol min}^{-1}$ (mg cell protein)⁻¹, respectively. The growth rate of this organism in synthetic medium containing less than 0.2 mM K⁺ was dependent on K⁺ concentration and was half-saturated at about 50 μM K⁺. The cells grown at low concentrations of K⁺ induced another K⁺ transport system with K_m and V_{max} values of 0.3 mM and 0.6 $\mu\text{mol min}^{-1}$ (mg cell protein)⁻¹, respectively. The high-affinity system appeared when cells were grown at concentrations less than 2.0 mM K⁺ and was fully induced at 0.1 mM K⁺ and below. A mutant strain (FS181) unable to grow at 0.1 mM K⁺ was isolated and found to be defective in the inducible K⁺ transport system.

Keywords: *Vibrio alginolyticus*, potassium transport, inducible ion transport

INTRODUCTION

Potassium ions have two important cellular functions; the activation of a number of enzymes and the maintenance of cell osmolarity (Walderhaug *et al.*, 1987). Since the only way to supply K⁺ is by accumulation, K⁺ transport is indispensable for all living organisms. K⁺ transport by *Escherichia coli* has been well characterized (Epstein & Davies, 1970; Epstein & Kim, 1971; Dosch *et al.*, 1991; Bakker, 1992a). *E. coli* has constitutive low-affinity and inducible high-affinity K⁺ transport systems. The inducible high-affinity K⁺ transport system, designated Kdp ($K_m = 2 \mu\text{M}$), comprises three proteins encoded by the *kdpABC* operon, and a Kdp-like system has been detected in many Gram-negative bacteria (Hesse *et al.*, 1984; Walderhaug *et al.*, 1989). The constitutive K⁺ transport systems, TrkD (KupD), TrkG, TrkH and TrkF, have also been recognized (Bossemeyer *et al.*, 1989; Schlösser *et al.*, 1991; Bakker, 1992b). Except for TrkF, the other systems are well characterized and belong to low-affinity K⁺ transport systems with apparent K_m values of 0.3–3 mM (Dosch *et al.*, 1991). The existence of constitutive low-affinity and inducible high-affinity K⁺ transport systems have been reported in *Paracoccus denitrificans* (Erecinska *et al.*, 1981), *Bacillus acidocaldarius* (Bakker *et al.*, 1987) and *Rhodobacter sphaeroides* (Abee *et al.*, 1992).

In contrast, K⁺ transport by marine bacteria has not been investigated in detail. Hassan & MacLeod (1975) reported an Na⁺-dependent K⁺ uptake system in *Alteromonas haloplanktis* and the importance of K⁺ uptake for the regulation of cytoplasmic pH in a marine *Vibrio alginolyticus* was demonstrated (Tokuda *et al.*, 1981). *V. alginolyticus* has a respiration-driven Na⁺ pump (Tokuda & Unemoto, 1982), a K⁺/H⁺ antiporter that functions as a regulator of cytoplasmic pH (Nakamura *et al.*, 1984, 1992) and an electrogenic Na⁺/H⁺ antiporter (Nakamura *et al.*, 1992). Since K⁺ uptake has been shown to play a central role in the regulation of cellular K⁺, Na⁺ and H⁺, its properties were studied in this paper. In addition to a constitutive low-affinity K⁺ transport system, this organism was found to have an inducible K⁺ transport system, which does not resemble those reported in *E. coli* and other bacteria.

METHODS

Bacterial strain and growth media. *V. alginolyticus* strain 138-2 was grown aerobically at 37 °C in a synthetic medium composed of 0.3 M NaCl, 2 mM Na₂HPO₄, 15 mM (NH₄)₂SO₄, 10 μM FeSO₄, 5 mM MgSO₄, 1% (w/v) glycerol, 50 mM Tris/HCl (pH 7.5) and the desired concentration of KCl. In the absence of added KCl, the above medium contained about 5 μM K⁺ as a contaminant. To determine the K⁺-dependent growth at low K⁺ concentrations, precultured cells

were washed twice with synthetic medium without K^+ by centrifugation before inoculating a K^+ -limited fresh medium. Culture growth was monitored by measuring OD_{600} in a Perkin-Elmer model 35 spectrophotometer and the growth rate was expressed as the growth rate constant defined as $0.693/\text{doubling time in h}$.

Preparation of K^+ -depleted cells. Unless otherwise noted, K^+ -depleted cells were prepared by a Tris-EDTA treatment according to Stewart *et al.* (1985). The cells were harvested at the late exponential phase of growth, washed twice with, and suspended in 0.5 M Tris/HCl (pH 8.0). The suspension was incubated for 1 min at 37 °C, and then sodium EDTA (pH 8.0) was added to a final concentration of 1 mM. After incubation for another 2 min at 37 °C, the cells were washed once with 0.5 M Tris/HCl (pH 8.0) and twice with synthetic medium lacking K^+ and glycerol. The cells were resuspended in the above synthetic medium at a hundredth volume of the growth medium. The cellular K^+ concentration was reduced to less than 10 nmol (mg cell protein) $^{-1}$, which corresponded to 3 mM. Protein was determined by the method of Lowry with bovine serum albumin as a standard.

The replacement of cellular cations with Na^+ (or Li^+) was performed under iso-osmotic conditions as described previously (Nakamura *et al.*, 1982). Briefly, harvested cells were treated twice with 0.4 M NaCl (or LiCl) containing 50 mM diethanolamine hydrochloride (pH 8.5) for 10 min at 25 °C. The K^+ -depleted and diethanolamine-loaded cells were treated twice with 0.4 M NaCl (or LiCl) containing 50 mM HEPES/NaOH (or HEPES/LiOH) at pH 7.0. By these treatments, the cellular K^+ was reduced to less than 3 mM and the cells contained about 500 mM Na^+ (or Li^+).

The depletion of cellular K^+ by hypotonic treatment (Mg^{2+} -washed cells) was performed as described by Hassan & MacLeod (1975). Harvested cells were treated twice with 50 mM $MgSO_4$ and resuspended in the same medium. By these treatments, the cellular Na^+ and K^+ were reduced to 3 and 40 mM, respectively.

Measurement of K^+ uptake. A filtration method (Nakamura *et al.*, 1982) was used for the measurement of initial velocity and accumulation of K^+ uptake. The reaction was carried out at 37 °C in synthetic medium containing different concentrations of K^+ at a cell density of 0.8–1.0 mg cell protein ml^{-1} . K^+ was determined by flame photometry using a Perkin-Elmer 403 atomic absorption spectrophotometer.

Isolation of mutants unable to grow at low K^+ . Cells of *V. alginolyticus* were treated with 10 μg NTG ml^{-1} for 20 min at 37 °C in a medium containing 0.4 M NaCl, 44 mM maleic acid and 50 mM Tris (pH 6.0). NTG was removed by washing with the above buffer. About 70% of the cells were killed by this treatment. NTG-treated cells were cultured overnight at 30 °C with gentle shaking in a medium containing (all w/v) 0.1% yeast extract, 0.1% polypeptone, 1% glycerol and 50 mM Tris/HCl (pH 8.0) supplemented with 0.1 M NaCl and 0.4 M KCl (medium A). The cells were washed with the above complex medium supplemented with 0.5 M NaCl (medium B), which contained about 0.7 mM K^+ . The cells were grown in medium B at 37 °C for 1 h, 100 μg aztreonam ml^{-1} , a penicillin analogue, was then added to the culture and incubated for 2 h. The cells were washed with, and suspended in medium A and were incubated at 37 °C for 6 h. The above aztreonam treatment was repeated. The cells were finally plated on medium A agar plates. A mutant, FS181, was selected by replica plating from colonies that were able to grow in synthetic medium containing 2.0 mM, but not 0.1 mM K^+ .

RESULTS AND DISCUSSION

Effects of K^+ on the growth and K^+ uptake of *V. alginolyticus*

When the marine *V. alginolyticus* was cultured in synthetic medium, more than 0.2 mM K^+ was required for optimal growth (Fig. 1). At concentrations below 0.2 mM, the growth rate of this organism was dependent on external K^+ concentration. The K^+ -dependent growth rate was not influenced much by the concentration of K^+ in the preculture, and the growth rate was half-saturated at about 50 μM K^+ . Thus, compared with *E. coli* (Epstein & Davies, 1970), *V. alginolyticus* required relatively high concentrations of K^+ for optimal growth. Furthermore, the K^+ -dependent growth observed here was very similar to that of a Kdp-deficient mutant (Frag 5) of *E. coli* (Epstein & Davies, 1970), suggesting the absence of an inducible Kdp-like high-affinity K^+ transport system in this organism. The lag phase time required to grow at low K^+ was also unaffected by the K^+ concentration of the preculture (data not shown).

To examine possibilities of an induction of K^+ transport system in the cells grown at low K^+ , uptake of K^+ by this organism was compared with the cells precultured at 35 μM , 0.69 mM and 140 mM K^+ . When the uptake of K^+ was assayed at 1.0 mM K^+ , the cells precultured at lower K^+ exhibited a faster initial rate of K^+ uptake and a higher extent of K^+ accumulation (Fig. 2). However, when assayed at 10 mM K^+ , these cells accumulated K^+ to nearly the same extent. Since K^+ accumulation reached a steady state in 3 min, the effects of external K^+ on the extent of K^+ accumulation were examined with the cells precultured at 25 μM and 50 mM K^+ (Fig. 3). The cells precultured at 25 μM K^+ showed a high ability to accumulate K^+ at low external K^+ . These results implied

Fig. 1. Effects of K^+ on the growth rate of *V. alginolyticus*. Cells were precultured in synthetic medium containing 50 mM (■), 15 mM (●) or 20 μM (▲) K^+ , and then grown in synthetic medium containing different concentrations of K^+ . The growth rate constant was calculated as described in Methods.

Fig. 2. Time courses of K⁺ uptake by cells precultured in synthetic medium containing 35 μM (○, ●), 0.69 mM (△, ▲) or 140 mM (□, ■) K⁺. Cellular K⁺ was depleted by Tris-EDTA treatment. K⁺ uptake was measured in synthetic medium containing 1.0 mM (closed symbols) or 10 mM (open symbols) K⁺.

Fig. 3. Accumulation of K⁺ by cells precultured in synthetic medium containing 25 μM (▲) or 50 mM (●) K⁺. Cellular K⁺ was depleted by Tris-EDTA treatment. The amounts of K⁺ accumulated in 3 min were determined at different K⁺ concentrations.

an induction of a high-affinity K⁺ transport system in the cells precultured at low K⁺.

Since the cells precultured at 0.69 mM K⁺ showed an intermediate rate of K⁺ uptake in the presence of 1.0 mM K⁺ (see Fig. 2), a partial induction of the high-affinity K⁺ transport system was assumed. From the results of Figs 2 and 3, the induction of the high-affinity system was reflected by the difference in the extent of K⁺ accumulation measured in the presence of 1.0 mM and

Fig. 4. Effects of K⁺ in the preculture on the induction of the high-affinity K⁺ transport system. The cells were precultured at different K⁺ concentrations, and then cellular K⁺ was depleted by Tris-EDTA treatment. The amounts of K⁺ accumulated in 3 min were measured in the presence of 1.0 mM and 10 mM K⁺, and the ratio (1 mM K⁺/10 mM K⁺) was plotted against the K⁺ concentration in the preculture.

10 mM K⁺. Thus, the ratio of K⁺ accumulation at 10 mM K⁺ to that at 1.0 mM K⁺ was determined with the cells precultured at different K⁺ concentrations (Fig. 4). The high-affinity system appeared when grown at concentrations less than 2.0 mM K⁺, and it was fully induced at 0.1 mM K⁺ and below.

The initial rate of K⁺ uptake increased with the increase in external K⁺ and the activation curve conformed to Michaelis-Menten kinetics (Fig. 5). With cells precultured at 15 mM K⁺, the apparent K_m value for K⁺ and the maximum velocity (V_{max}) were calculated to be 3.0 mM and 1.5 μmol min⁻¹ (mg cell protein)⁻¹, respectively. When Rb⁺ was used instead of K⁺, the apparent K_m value for Rb⁺ and V_{max} were estimated to be 10 mM and 0.43 μmol min⁻¹ (mg cell protein)⁻¹, respectively. In contrast, with the cells precultured at 0.2 mM K⁺, the apparent K_m value for K⁺ and V_{max} were calculated to be 0.3 mM and 0.6 μmol min⁻¹ (mg cell protein)⁻¹, respectively (Fig. 5). These cells could also take up Rb⁺ with an apparent K_m value of 2 mM and V_{max} of 0.35 μmol min⁻¹ (mg cell protein)⁻¹. Cs⁺ uptake, however, was not observed with the cells precultured at any K⁺ concentration.

Thus, cells grown at high K⁺ exhibited a constitutive low-affinity K⁺ transport system, which was similar to the TrkH system of *E. coli* with respect to the K_m value (Dosch *et al.*, 1991). When grown at low K⁺, a high-affinity system could be detected. Although the induced system showed a higher affinity for K⁺ than that of the constitutive one, its K_m value was still very large compared to an inducible Kdp-type high-affinity system with a K_m value of 2 μM reported in *E. coli* and other

Fig. 5. Double-reciprocal plots of initial velocity versus K^+ concentration with cells precultured in synthetic medium containing 15 mM (●) or 0.2 mM (▲) K^+ . Cellular K^+ was depleted by Tris-EDTA treatment. The initial velocity of K^+ uptake (V) was determined at different K^+ concentrations and was expressed in $\mu\text{mol min}^{-1} (\text{mg cell protein})^{-1}$.

bacteria (Erecinska *et al.*, 1981; Hesse *et al.*, 1984; Bakker *et al.*, 1987; Abee *et al.*, 1992). Moreover, the induced system actively took up Rb^+ in contrast to the Kdp system of *E. coli* (Bossemeyer *et al.*, 1989). These results implied that the induced system did not resemble to Kdp-type K^+ -translocating ATPase.

Properties of the K^+ transport system

The marine bacterium, *A. haloplanktis* was reported to require Na^+ for active uptake of K^+ and the cotransport of K^+ with Na^+ was proposed (Hassan & MacLeod, 1975). In the Hassan & MacLeod (1975) experiments, K^+ -depleted cells were prepared by suspending the cells in 50 mM MgSO_4 , and thus the cells were plasmolysed. To examine the effect of plasmolysis on K^+ uptake, K^+ -depleted cells prepared by different methods were compared (Table 1). When K^+ -depleted cells were prepared under hyptonic conditions (Mg^{2+} -washed cells), external Na^+ was required for K^+ uptake. The light scattering at 500 nm of the suspension of Mg^{2+} -washed cells decreased by more than 20% from the original value during K^+ uptake, indicating the occurrence of deplasmolysis by K^+ accumulation. In contrast, K^+ -depleted cells prepared under iso-osmotic conditions actively took up K^+ without significant changes in light scattering of the cell suspension and even in the absence of Na^+ (the Li^+ -loaded cells in LiCl medium). Thus, Na^+ was not obligatory for the active uptake of K^+ . Similar results were described in *E. coli* (Bakker *et al.*, 1984). Extrusion of some cations is necessary for the accumulation of K^+ . Since the Li^+ -loaded cells in LiCl medium showed lower activity, Na^+ was apparently better than Li^+ as a counter cation for K^+ uptake.

Table 1. Comparison of the initial rate of K^+ uptake by K^+ -depleted cells of *V. alginolyticus* prepared by different methods

The cells were precultured at 15 mM K^+ , and then K^+ -depleted/ Na^+ (Li^+)-loaded cells were prepared under iso-osmotic conditions as described in Methods. The depletion of cellular K^+ by hypotonic treatment (Mg^{2+} -washed cells) was performed as described in Methods. The initial rate of K^+ uptake was assayed in 0.4 M-NaCl, 50 mM-HEPES/NaOH, pH 7.0, (NaCl medium), or 0.4 M-LiCl, 50 mM-HEPES/LiOH, pH 7.0, (LiCl medium), containing 20 mM glycerol and 10 mM K^+ .

K^+ -depleted cells	K^+ uptake [$\mu\text{mol min}^{-1}$ ($\text{mg cell protein})^{-1}$] in:	
	NaCl medium	LiCl medium
Na^+ -loaded cells	0.82	0.80
Li^+ -loaded cells	0.81	0.37
Mg^{2+} -washed cells	0.23	0.01

In most of our experiments, cellular K^+ was depleted by the Tris-EDTA treatment described in Methods. Since this method was relatively simple and the K^+ -depleted cells always showed a high K^+ transport activity, it was used throughout the experiments.

Isolation of a mutant defective in the inducible K^+ transport system

NTG-induced mutants were enriched by using aztreonam as described in Methods. A mutant strain, FS181, was isolated which was unable to grow in the synthetic medium containing 0.1 mM K^+ on an agar plate. The rate of reverse mutation of FS181 was estimated to be about 10^{-6} – 10^{-7} , suggesting that FS181 was obtained by a single mutation.

The wild type and mutant strain FS181 were precultured in synthetic medium containing 0.2 mM K^+ and their abilities to accumulate K^+ were compared (Fig. 6). Under those conditions, the wild type cells accumulated K^+ at 0.75 mM, indicating the induction of the high-affinity K^+ transport system (compare with Fig. 3). In contrast, the mutant strain FS181 was unable to accumulate K^+ at 0.75 mM, implying that it was defective in the induction of the high-affinity system. The apparent K_m value and V_{max} of FS181 grown at 0.25 mM K^+ were estimated to be 4 mM and $0.24 \mu\text{mol min}^{-1} (\text{mg cell protein})^{-1}$, respectively. Strain FS181 apparently lost the high-affinity system, but the V_{max} of the constitutive K^+ transport system was also reduced. Thus, growth at low K^+ seems to either inhibit or repress the low-affinity system. Alternatively, since K^+ transport systems are generally composed of multi-subunits in *E. coli* (Bakker, 1992b), a deficiency in one component may possibly affect another K^+ transport system. The mutant isolated in this paper will help to further characterize these K^+ transport systems.

Fig. 6. Accumulation of K⁺ by cells precultured in synthetic medium containing 0.2 mM K⁺. Accumulation of K⁺ by the wild type (●) or strain F5181 (▲) were determined as described in the legend to Fig. 3.

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