The Na^+ and K^+ transport deficiency of an *E. coli* mutant lacking the NhaA and NhaB proteins is apparent and caused by impaired osmoregulation

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Abstract Cells of the *E. coli* mutant EP432, which lacks the two Na^+/H^+ antiporters, NhaA and NhaB, have been reported to have an impaired sodium transport activity (Harel-Bronstein et al. (1995) J. Biol. Chem. 270, 3816–3822). Here we report that active transport of Na^+ in EP432 cells can be restored to wild-type levels, either by a high K^+ concentration or by an increase in the medium osmolarity. We suggest that this mutant is primarily deficient in osmoregulation rather than in cation transport per se.

Key words: Sodium/proton antiporter; Sodium transport; *Escherichia coli*

1. Introduction

Two membrane proteins catalyzing electrogenic Na^+/H^+ antiport, NhaA and NhaB, are widely believed to be the key catalysts of active Na^+ transport in *E. coli* cells (for a review, see [1]). The mutant with the double deletion, Δ*nhaA*,Δ*nhaB* (EP432), has been isolated and characterized [2,3]. The EP432 strain did not grow on some substrates which are symported with Na^+, such as proline and melibiose. Growth of the strain was inhibited by Na^+ and the Na^+/H^+ antiport activity in membrane vesicles was absent [2], which is consistent with the suggestion that NhaA and NhaB are the two main systems responsible for active Na^+ transport in *E. coli* cells. However, other findings are contradictory. EP432 cells cannot maintain as low an intracellular Na^+ concentration as wild type, but they still possess a $\Delta\bar{\mu}_{H^+}$-dependent Na^+ transport system [3]. It has also been reported that an *E. coli* mutant with the *nhaA* gene deleted and an impaired *nhaB* gene was capable of uphill Na^+ transport like wild type, but only at neutral pH [4]. However, in this case there was a possibility that a point mutation in *nhaB* did not destroy it but rather altered its properties. On the other hand, our recent measurements of Na^+ transport, and of ΔpH and $\Delta\psi$ in *E. coli* cells and membrane vesicles led us to conclude that uphill efflux of Na^+ can hardly be explained by the operation of the electrogenic Na^+/H^+ antiporters NhaA and NhaB, but rather by a different system (called AST) that is more tightly coupled with respiratory chain complexes [5,6].

The purpose of this work was to measure uphill Na^+ efflux in the Δ*nhaA*,Δ*nhaB E. coli* mutant EP432, and to compare it with wild-type transport. Our findings indicate that this mutant is impaired in the regulation of cytoplasmic cation levels (osmoregulation), but that it retains the active sodium transport system.

2. Materials and methods

2.1. Strains and growth conditions

The *E. coli* strains TA15 and EP432 (*mel*BLid, Δ*nhaA1*,Δ*nhaB1*, Δ*lacZY*, *thr1*) were a gift from Dr. E. Padan, Hebrew University, Jerusalem. For cation transport measurements the cells were grown in rich medium containing 0.5% tryptone, 0.5% yeast extract, 100 mM KCl, 100 mM NaCl and 50 mM tricine-KOH/NaOH, pH 8.4. For osmotic shock the cells were grown in a medium containing 0.5% tryptone, 0.5% yeast extract, 100 mM KCl and 6.5 mM Na^+ as a background (LBK). For growth experiments the cells were grown in a medium containing 0.5% tryptone, 0.5% yeast extract (LBns) (6–7 mM Na^+ and K^+ as a background level) and KCl or mannitol were added as described in the figure legends.

2.2. Cell treatment and measurements of Na^+ and K^+ content

The EP432 mutant was found to be much more sensitive than wild type to a decrease of osmolarity of the medium into which the cells were suspended. Even a very small dilution of the mutant cell suspension could lead to an irreversible loss of internal cations and probable shrinkage of the cells; this effect was much more pronounced in the first half of exponential growth phase. Thus, for transport measurements cells were harvested from the second half of the exponential phase. Also, in contrast to wild type, EP432 cells were found to be unstable during storage at room temperature, or on ice. The cells tended to lose their capability of uphill cation transport during 0.5–1 h of storage. To avoid this, 1-ml aliquots from the growing cell culture were sedimented in an Eppendorf microcentrifuge, at 13 000 rpm for 4 min, washed once with Na^+-medium (150 mM NaCl, 50 mM tricine-NaOH, 8.4), resuspended in the same medium and used for measurements immediately. Only cells from a growing culture are capable of transporting alkali cations. Since EP432 cells growing at high Na^+ contained significant amounts of Na^+ in the cytoplasm ([3], present data), K^+ could be replaced by Na^+ just by washing with Na^+ medium. There was no necessity for a special procedure to load these cells with Na^+. Parent cells, TA15, were loaded with Na^+ using diethanolamine treatment [7], and suspended in Na^+ medium. EP432 mutant and wild-type cells were not depleted in endogenous substrates and added substrates did not improve the observed Na^+ transport.

For osmotic shock experiments 1-ml aliquots of a growing cell culture (LBK) were sedimented in an Eppendorf centrifuge, resuspended in 50 μl of the same growth medium, and immediately diluted into 1 ml of growth medium without added KCl (LBns).

Sodium and potassium inside the cells were determined by flame photometry as previously described [8], except that the cells were washed on a filter with 0.8 M mannitol.

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3. Results and discussion

Respiration-dependent active transport of alkali cations in mutant and wild-type *E. coli* cells incubated in Na^+ medium was initiated by the addition of 10 mM KCl. Fig. 1A shows that both Na^+ extrusion and K^+ accumulation were insignificant in the mutant cells as compared to wild type. However, transport activity in the mutant could be restored by two different modifications of the experimental conditions: (i) by initiating transport with 100 mM K^+ instead of 10 mM (Fig. 1B), or (ii) by supplementing the medium with 0.4 M mannitol (Fig. 1C). In both these cases the observed active efflux of Na^+ was similar in the mutant and in the parent TA15 cells, although the total potassium accumulation was slightly higher in the latter case.

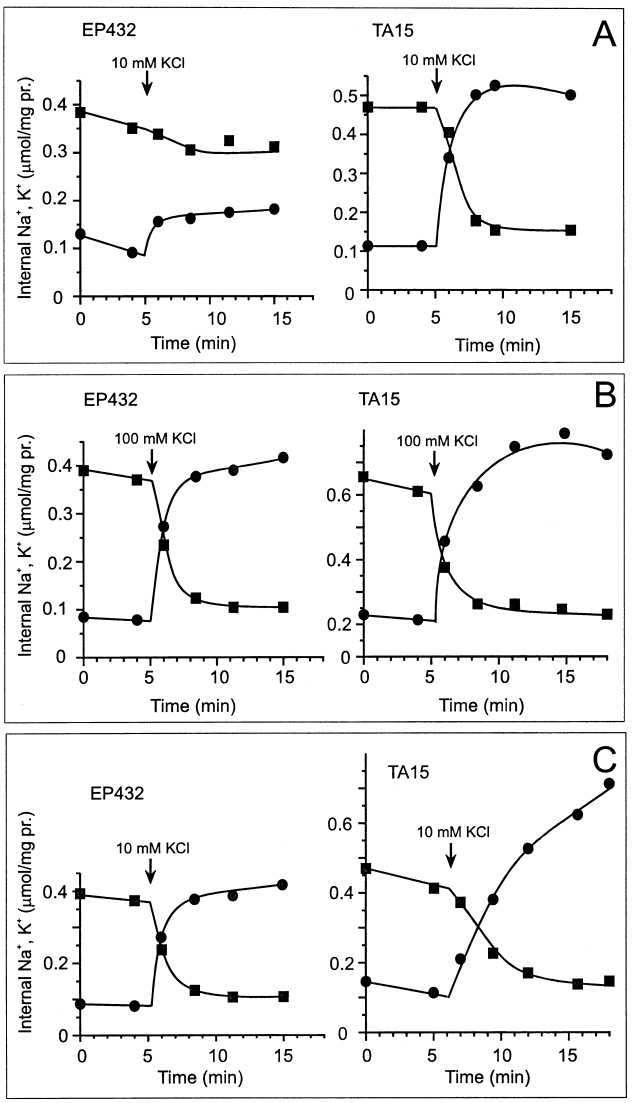

Fig. 1. Active Na^+ and K^+ transport in the EP432 mutant strain can be restored to wild-type levels by a high K^+ concentration or high medium osmolarity. Na^+-loaded EP432 and TA15 cells were diluted into medium containing 150 mM NaCl, 50 mM Tricine-NaOH, pH 8.4 at zero time, and uphill cation transport was initiated by KCl addition as shown. A: 10 mM KCl was added. B: 100 mM KCl was added. C: 10 mM KCl was added and the dilution medium was supplemented with 0.4 M mannitol. Circles: Intracellular content of K^+. Squares: Intracellular content of Na^+.

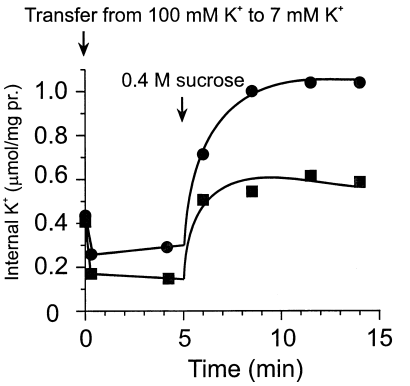

Fig. 2. Response of the intracellular K^+ content to variation of medium osmolarity. EP432 (squares) and TA15 (circles) cells growing in LBK were transferred into LB without added KCl at zero time. At the moment marked by the arrow the cell suspension was diluted twice with LB containing 0.8 M sucrose.

These experiments show that in the EP432 mutant there is a strict dependence of alkali cation transport on the concentration of K^+ or the osmolarity of the medium. This suggests that there is a disorder of osmoregulation in the mutant rather than a defect in the active sodium transport machinery itself. It seems that K^+ transport is blocked in the mutant cells at low osmolarity, and since Na^+ transport requires the influx of K^+ as a counter-ion, the loss of K^+ transport leads secondarily to loss of Na^+ transport.

Next, we addressed the reason for the apparent K^+ transport deficiency in the mutant cells. Since none of the known three main K^+ transport systems were changed genetically in this mutant, the only reasonable explanation is loss of proper regulation of K^+ transport. The known highly active potassium influx system, Trk, should be expressed under the used growth conditions, and needs to be strictly regulated to avoid a futile cycle [9]. The Trk system is known to be under strong control by cell turgor pressure: at high turgor pressure the V_{max} of Trk is decreased by two orders of magnitude in comparison to K^+-depleted cells [10,11]. In growing cells the activity of Trk is low [9]; thus its default state is a suppressed one, and it needs to be activated for fast K^+ accumulation. Distortion of a sensoring and/or transmission turgor signal would thus prevent the activation of Trk, and block the accumulation of potassium.

To find out more directly whether the osmosensitivity of the EP432 mutant cells does actually differ from that of wild type, we used the down- and upshock model in which *E. coli* cells are exposed to a fast change of medium osmolarity (for a review, see [12]). After growth in LBK medium the *E. coli* cells were transferred into LB medium with lower osmolarity (no added KCl) and then 0.4 M sucrose was added. Fig. 2 shows a clear difference between mutant and parent strains in this respect. Initially the mutant cells lose more K^+ than wild type due to a slight hypo-osmotic shock. The subsequent hyper-osmotic shock induces accumulation of K^+ which occurs to a much lower extent in the mutant than in the parent strain.

Another indication of distorted osmoregulation in the EP432 mutant is its inability to grow in LB medium at low [K^+] (approx. 6 mM K^+). As shown in Fig. 3, this can be overcome either by an increase in K^+ concentration, as already described by Harel-Bronstein et al. [3], or an increase

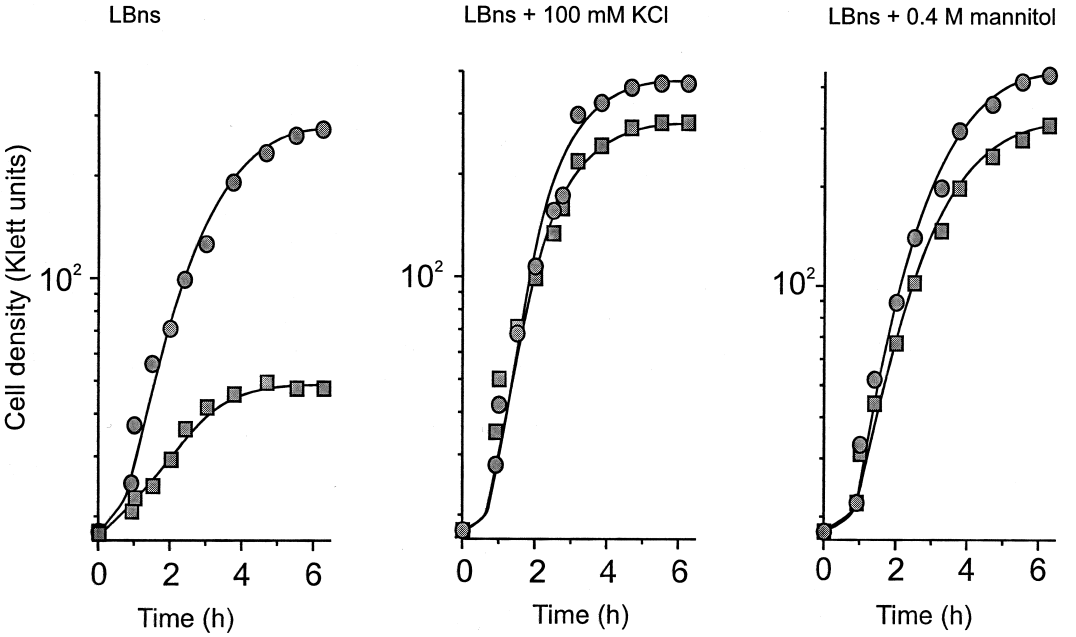

Fig. 3. Dependence of cell growth on potassium and mannitol. EP432 (squares) and TA15 (circles) cells were grown in LBns medium with or without addition of 100 mM K^+ or 0.4 M mannitol. The LBns medium contained approximately 8 mM K^+ and 6 mM Na^+ as a contamination.

of medium osmolarity. The growth of either strain in the presence of a high K^+ concentration was independent on mannitol supplementation (not shown).

Active transport of alkali cations in bacterial cells does not depend only on driving force and the transport catalyst per se. As long as transport is measured in the whole organism, it will also depend on the current cation status of the bacteria maintaining cell turgor, which in turn is governed by sophisticated regulation. A change in this regulation may therefore cause secondary changes in measured transport characteristics that are observable only under some experimental conditions. Therefore an observed lack of transport activity in a mutant is at most an indication, but certainly no proof that it is the gene encoding the cation transporter that has been mutated.

If the transport system is expressed, but either inhibited or not activated due to the mutation, its activity might nevertheless be recovered in membrane vesicles. The EP432 mutant lacks two Na^+/H^+ antiporters, NhaA and NhaB, and was reported to have lost Na^+ transport capacity on the cell level [2,3]. However, we found previously that respiration-dependent uphill Na^+ transport did not differ significantly in mutant and wild-type membrane vesicles [6], even though the Na^+/H^+ antiporter activity was negligible in mutant strain membranes [2,3]. The results presented in this paper clearly show that the EP432 mutant cells are fully capable of active Na^+ extrusion. The Na^+-transport system is expressed in the mutant, but its operation is inhibited by secondary events during cell growth, and could be re-activated under certain conditions.

Harel-Bronstein et al. [3] also described Na^+ excreting capacity of the EP432 mutant, but they concluded that this was due to another system much less efficient than NhaA and NhaB, because it created only low Na^+ gradients in the mutant cells. We have shown here that the operation of the Na^+-transport system in the EP432 strain is masked by an apparent cation transport deficiency, which is due to corruption of osmoregulation. This phenomenon is not dependent on Na^+ since it can be demonstrated at low concentration of Na^+ outside as well as inside of cells (K^+ loss due to dilution of cell suspension, requirement of increased osmolarity for growth of EP432 cells). This means that the function of NhaA and NhaB may not only be Na^+-sensoring as might be expected from the Na^+/H^+ antiporter activity, but that these proteins may be involved in osmoregulation in an indirect way. Corruption of *nhaA* causes an apparent loss of nhaR [13], which belongs to the LysR family of bacterial regulatory proteins [14], and could regulate transcription not only of *nha*A, but of other genes, too. On the other hand, either NhaA or NhaB might function as a sensor of Na^+ and the other as a sensor for sucrose and other osmolytes, or both of them could participate in signal transmission.

The results presented here show that *E. coli* cells are capable of full-scale active Na^+ transport in the absence of both NhaA and NhaB. Taken together with the previously reported significant sodium transport activity of membranes from the double mutated strain [6], this raises the question of the identity of the system responsible for transport. It could not be a primary Na^+ pump proposed to be expressed in the *ΔnhaA,ΔnhaB* mutant by second site mutation [3], or a primary Na^+ pump induced by conditions that lower $\Delta\bar{\mu}_{H^+}$ [15,16]. Irrespective of whether these Na^+ pumps are the same entity, the expression of both requires very specific growth conditions (more than 24 h incubation of EP432 cells in high Na^+ medium [3], or growth of wild type under conditions that lower the $\Delta\bar{\mu}_{H^+}$ [15,16], neither of which was required in the present work). In our hands the EP432 cells maintained their phenotype: Na^+ and K^+ gradients during growth were significantly lower than in wild-type cells. Another system which could participate in Na^+ transport in *E. coli* is ChaA suggested recently as an effective sodium transport system [17]. ChaA, which was first reported by Ivey et al. [18], possesses a significant sequence similarity to calsequestrin and has been shown to catalyse Ca^{2+}/H^+ antiport in membrane

vesicles. Its Na^+/H^+ activity in vivo was reported to be low and ChaA was proposed to be a Ca^{2+}/H^+ antiporter whose overexpression leads to resistance to growth inhibition by calcium and sodium [18]. However, Sakuma et al. [17] argued that the *E. coli* strain used by Ivey et al. had a mutation which reduces the ChaA activity. However, in the present work we used the same strain as Ivey et al., which makes it quite improbable that ChaA is responsible for the observed Na^+ efflux.

Other candidates for the protein catalysing Na^+ transport may be found by searching the complete *E. coli* genome for genes with similarity to well-known Na^+-transporting systems in eukaryotes. The search using the sequence analysis software BLASTP 1.4.11 [19] and the GenBank database revealed no genes with significant similarity to Na^+ channels but two genes were found with similarity to Na^+/H^+ exchangers. One of them in the *soxR-acs* intergenic region which encodes a hypothetical 60.5-kDa protein, described in GenBank as similar to eukaryotic Na^+/H^+ exchangers. The other encodes a 536 amino acid ORF, P26434, described in GenBank as similar to the 720 amino acid protein NAH_RAT. These proteins are not unique for *E. coli*, since we also found related genes in the genomes of two eubacterial and two archebacterial species. These genes products might thus operate as Na^+/H^+ exchangers and participate in sodium transport. However, they are not related to the *nhaA*, *nhaB*, or *chaA* genes of *E. coli* [17], nor to *nhaC* of *Bacillus firmus* [20] or *nhaD* of *Vibrio parahaemolyticus* [21], of which the latter improved the Na^+-sensitive phenotype of the *nha*A and *nha*B-deficient *E. coli* mutant.

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