Biochimica et Biophysica Acta, 1143 (1993) 62–66

BBABIO 43820

ATP-driven potassium transport in right-side-out membrane vesicles via the Kdp system of *Escherichia coli*

Ralf Kollmann and Karlheinz Altendorf

Universität Osnabrück, Fachbereich Biologie / Chemie, Arbeitsgruppe Mikrobiologie, Osnabrück (Germany)

(Received 17 August 1992)
(Revised manuscript received 26 November 1992)

Key words: Kdp-ATPase; Potassium transport; Right-side-out membrane vesicle; Phosphoglycerate transport; ATP generation; (*E. coli*)

The ATP-generating system described by Hugenholtz, J., Hong, J.-S. and Kaback, H.R. ((1981) Proc. Natl. Acad. Sci. USA 78, 3446–3449) has been used to synthesize ATP up to 1.8 mM in right-side-out membrane vesicles from *Escherichia coli*. This ATP level was sufficient to drive uptake of potassium ions via the Kdp-ATPase. In the *kdp* wild type strain about 110 nmoles K^+/mg membrane protein were accumulated. This process was still partially sensitive to the well-known inhibitors of P-type ATPases, orthovanadate and bafilomycin B_1.

Introduction

Potassium ions (K^+) serve important functions in bacteria: (i) they activate cytoplasmic enzymes [1], (ii) they are a major contributor to cell turgor [2], (iii) they are probably involved in cytoplasmic pH homeostasis via K^+-H^+ antiporters [3,4], and (iv) they play a role in the regulation of gene expression during osmoadaptation [5,6]. In order to satisfy the cells' need for K^+ within this intricate regulatory network, K^+ transport in *Escherichia coli* is catalyzed by constitutive and inducible systems, influx and efflux systems, by a primary pump as well as secondary porters and stretch-activated channels (for review, see Ref. 7).

Apart from the constitutive K^+ uptake systems Kup, TrkH, and TrkG [8,9] the cells possess the inducible Kdp system. Its intricate regulation at the levels of both transcription and enzyme activity [2], and its extraordinarily high affinity (K_m for K^+ uptake: 2 μM) [10] and specificity [11] for K^+ ions all suggest that Kdp functions as an emergency system. The *kdp* genes have been cloned and two separate transcriptional units have been identified [12]. The *kdpABC* operon codes for the three cytoplasmic membrane proteins KdpA (58 189 Da), KdpB (72 112 Da), and KdpC (20 267 Da) [13]. The second operon *kdpDE* encodes the regulatory proteins KdpD (98 656 Da) and KdpE (25 240 Da) [14], which belong to a class of sensor/regulator systems, formerly called 'two component system' [15].

An ATPase activity reflecting the properties of the transport system was found to be associated with membranes of Kdp-induced cells [16,17], confirming that ATP is the driving force for K^+ uptake via the Kdp system [18]. The enzymatic properties and the inhibition characteristics of the purified Kdp-ATPase [19–21] are strikingly similar to those of cation-translocating ATPases of eukaryotic cells [22], which belong to the so-called P-type ATPases [23], forming a covalent phosphorylated intermediate as part of the reaction cycle. Despite the fact that the Kdp-ATPase has genetically, physiologically, and biochemically been well defined, information about the energetics of K^+ transport is still rather scarce. This is partly due to the difficulty to measure ion movements via Kdp given the complexity of an intact cell. Furthermore, reconstitution of the purified Kdp complex in liposomes has only been partially successful; ATPase activity has been demonstrated, but K^+ transport capacity is still lacking (R. Kollmann and K. Altendorf, unpublished results). Therefore, we have used membrane vesicles, which have proven to be a useful tool to study active transport. Here we report for the first time that right-side-out vesicles are capable of K^+ uptake provided that ATP is generated inside.

Correspondence to: K. Altendorf, Universität Osnabrück, Fachbereich Biologie/Chemie, Arbeitsgruppe Mikrobiologie, Barbarastraße 11, D-4500 Osnabrück, Germany.

Materials and Methods

Chemicals

All chemicals used were of analytical grade. Pyruvate kinase, sodium phospho*enol*pyruvate, lithium D-lactate, luciferase/luciferin extract and valinomycin were purchased from Sigma (Deisenhofen, Germany). Orthovanadate was obtained from BDH Chemicals (Pool, UK) and L-[U-^{14}C]proline was purchased from Amersham (Braunschweig, Germany). Bafilomycin B_1 was kindly provided by Bayer (Wuppertal, Germany).

Bacterial strains and growth conditions

The *E. coli* K12 derivatives TK2240–40 (*thi, rha, lacZ, nagA, trkA405, trkD1*, F'*kdp*$^+$) and TK2281 (*thi, rha, lacZ, nagA, trkA405, trkD1, ΔkdpABCDE*) were kindly provided by Dr. W. Epstein (University of Chicago, USA) [16,24]. The respective *unc* derivatives TKA1000 and TKR2000 were constructed by introducing the *atp-706* (ΔIBEFHA) genotype via transduction with bacteriophage P1 [19]. Strains TK2240–40, TKA1000 and TKR2000 were transformed with plasmid pBR322-pgt2 [25], which was kindly supplied by Dr. H.R. Kaback (UCLA, USA). The minimal media used are identical to those described by Epstein and Davies [26]. The Kdp-system was induced under conditions as described previously by Siebers et al. [20]. Strain TKR2000 was grown in medium containing 115 mM potassium.

Preparation of membrane vesicles

The preparation of right-side-out membrane vesicles was carried out according to the lysozyme-EDTA method of Kaback [27] with the following modifications. Cells were grown to late logarithmic phase to an A_{610} of 0.5–0.7. The EDTA solution was adjusted to the desired pH with NaOH instead of KOH. The potassium phosphate buffer was replaced by a sodium phosphate buffer of the same ionic strength. The spheroplast suspension was transferred directly into 100–150 volumes of 50 mM sodium phosphate buffer (pH 6.6) containing 50 μg/ml pyruvate kinase and 5 mM ADP to meet the requirements for the ATP-regenerating system.

Assays

K^+ uptake in right-side-out vesicles was determined in a reaction mixture (0.2 ml) containing (final concentrations) 50 mM sodium phosphate buffer (pH 7.8), 10 mM $MgCl_2$, 0.15 mg of membrane protein, 1 mM KCl, 10 mM sodium phospho*enol*pyruvate and 20 mM lithium D-lactate, as indicated. With phospho*enol*pyruvate as the energy source, the samples were incubated for 30 min at 30°C before addition of KCl; with D-lactate as additional energy source, preincubation was carried out for 5 min. The reaction was terminated by the addition of 3 ml 100 mM lithium chloride, immediately filtered through a cellulose nitrate filter (0.45 μm pore size), and washed once with 3 ml 100 mM lithium chloride. The wet filters were transferred into 1 ml 5% TCA, frozen for at least 30 min at −20°C, thawed and boiled for 10 min. After the addition of 2 ml 7 mM cesium chloride, the potassium concentration was determined in a flame photometer.

To determine the ATP content of the right-side-out vesicles the firefly luciferase reaction was used [28]. The measurements were carried out according to the method of Chapman et al. [29] with some minor modifications. A reaction volume of 0.2 ml containing 0.15 mg of protein was used. 1 ml pre-chilled (4°C) 12% $HClO_4$ and 5 mM NaH_2PO_4 was added and the whole sample was stored for 30 min on ice. The sample was centrifuged for 10 min at 5000 rpm and the supernatant was adjusted to pH 7.0 by addition of 2 M KOH/0.3 M Mops. After precipitation of the protein (−20°C for 30 min) the sample was thawed and centrifuged for 10 min at 5000 rpm. 50 μl of the supernatant, 50 μl of the luciferase/luciferin extract and 900 μl 20 mM glycylglycine-buffer was used for the final ATP determination. The emitted light was measured in a luminometer (Berthold Luminomat LB 9500 T).

Proline uptake [25] was carried out in a reaction mixture (0.2 ml) containing (final concentrations) 50 mM sodium phosphate buffer (pH 6.6), 10 mM $MgSO_4$, 0.08 mg of membrane protein, 3.5 μM L-[U-^{14}C]proline (250 mCi/mmol) and either 20 mM lithium D-lactate or 10 mM sodium phospho*enol*pyruvate, as indicated. With D-lactate as the energy source, the samples were incubated for 15 min at 30°C before the addition of D-lactate. Incubation was continued for 5 min before the reaction was started by the addition of proline. With phospho*enol*pyruvate as the energy source, preincubation was carried out for 30 min. Uptake was terminated by the addition of 5 ml of 200 mM lithium chloride, immediately filtered through cellulose nitrate filters (0.45 μm pore size) and washed once with the same volume of lithium chloride. The filters were dried and counted in a liquid scintillation counter.

Results

Membrane vesicles from *Escherichia coli* retain the capacity to convert respiratory energy (i.e., provided by the addition of D-lactate) into a $\Delta\tilde{\mu}_{H^+}$, which in turn provides the energy for active transport. Since ADP is no longer available within the lumen of the vesicles, they are not able to synthesize ATP. In order to provide ATP as the energy source for K^+ transport via the Kdp-ATPase in right-side-out vesicles from *E. coli*, all strains used in this study have been transformed with plasmid pBR322-pgt2, coding for the phospho-

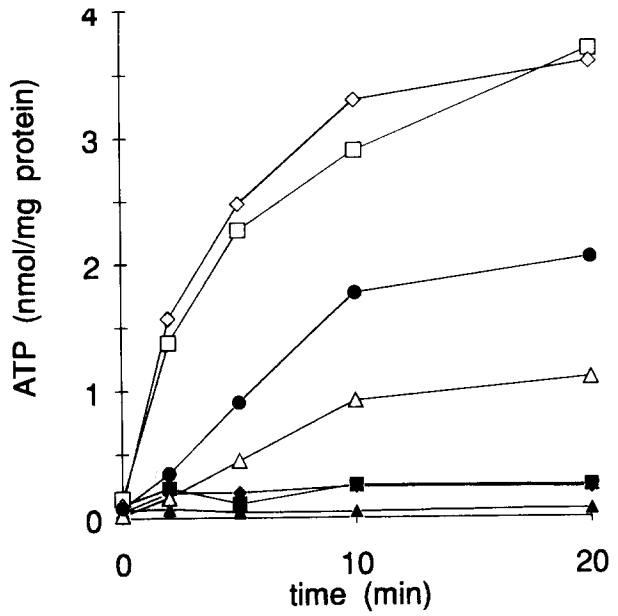

Fig. 1. Phospho*enol*pyruvate-dependent ATP generation in right-side-out membrane vesicles. Vesicles were prepared from *E. coli* TKA1000 (□——□, ■——■), TKR2000 (◇——◇, ◆——◆) and TK2240-40 (△——△, ▲——▲, ●——●), all transformed with plasmid pBR322-pgt2. Experimental details were otherwise as described in Materials and Methods. The protein concentration was 0.15 mg/200 μl. Samples were preincubated for 3 min and the reaction was started by the addition of PEP (10 mM) and D-lactate (20 mM). Symbols: □——□, ◇——◇, △——△, PEP; ■——■, ◆——◆, ▲——▲, no PEP; ●——●, PEP + D-lactate.

glycerate uptake system of *Salmonella typhimurium* [30]. This transport system also accepts phospho*enol*pyruvate (PEP) as a substrate. Therefore, PEP taken up from the medium, together with pyruvate kinase, ADP, and inorganic phosphate, which have been provided to the intravesicular space during lysis of the spheroplasts, constitute an ATP-generating system [25].

Two sets of experiments were used to test the functionality of the membrane vesicles. First, the capacity of the vesicles to synthesize ATP was tested. Upon uptake of PEP, synthesis of ATP was elicited in all vesicles derived from the *E. coli* strains TKA1000 (*Δunc*, *kdp*$^+$), TKR2000 (*Δunc*, *Δkdp*), and TK2240-40 (*unc*$^+$, *kdp*$^+$) (Fig. 1). In the latter case, however, the level of ATP was considerably lower due to the ATP-hydrolyzing activity of the ATP synthase. Furthermore, using membrane vesicles of TK2240-40, addition of both, PEP and D-lactate resulted in a somewhat higher ATP level. As expected, no ATP was synthesized in the absence of PEP and/or D-lactate. Second, the capacity of the vesicles to accumulate proline was investigated. Addition of D-lactate to membrane vesicles of TKA1000, TKR2000, and TK2240-40 resulted in the uptake of proline (Fig. 2). In contrast, when PEP was used as the energy source, transport of proline was only observed in vesicles of TK2240-40, but not in those derived from TKA1000 and TKR2000 both lacking the ATP synthase. The studies presented in Fig. 2 provide additional support for the argument that active

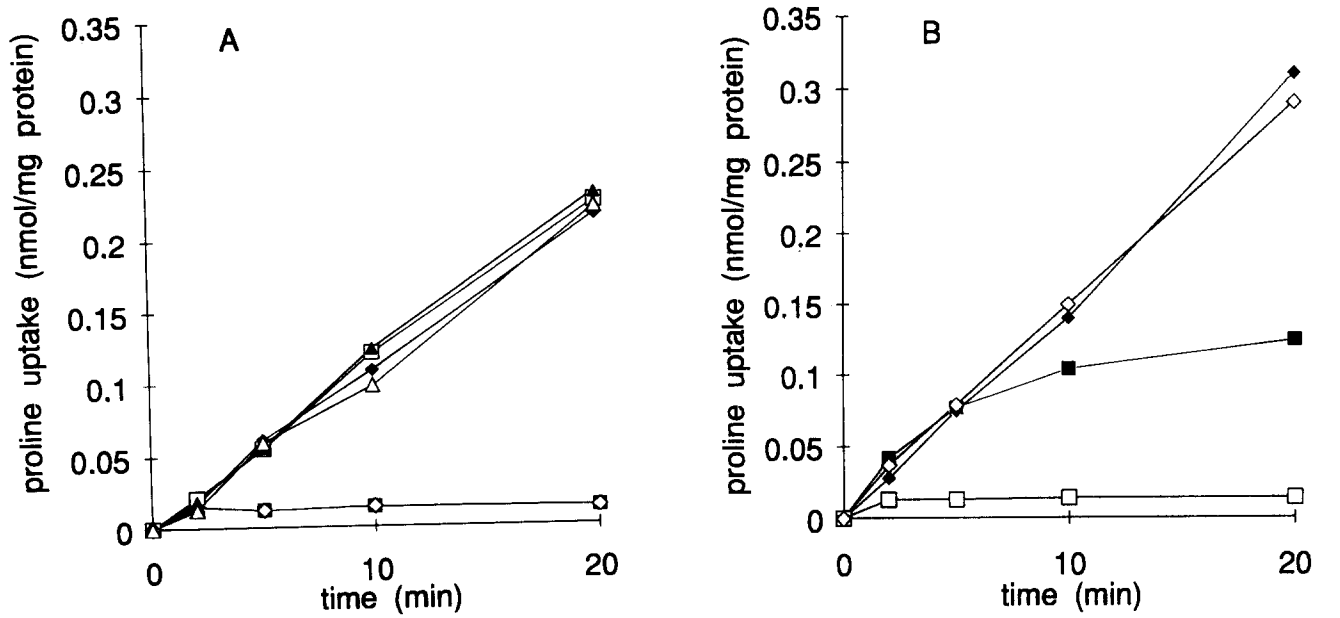

Fig. 2. Phospho*enol*pyruvate- or D-lactate-driven proline uptake by right-side-out membrane vesicles. (A) Vesicles were prepared from *E. coli* TKA1000 (■——■, PEP; □——□, D-lactate; ◆——◆, D-lactate + DCCD) and TKR2000 (◇——◇, PEP; ▲——▲, D-lactate; △——△, D-lactate + DCCD). (B) Vesicles were prepared from *E. coli* TK2240-40 (■——■, PEP; ◆——◆, D-lactate; □——□, PEP + DCCD; ◇——◇, D-lactate + DCCD). With PEP as the energy source samples were preincubated for 20 min before the reaction was started by the addition of proline. In the case of D-lactate the preincubation time was 5 min. DCCD (50 μM final concentration) was added 30 min before the addition of the energy source. Experimental details were otherwise as described in Materials and Methods.

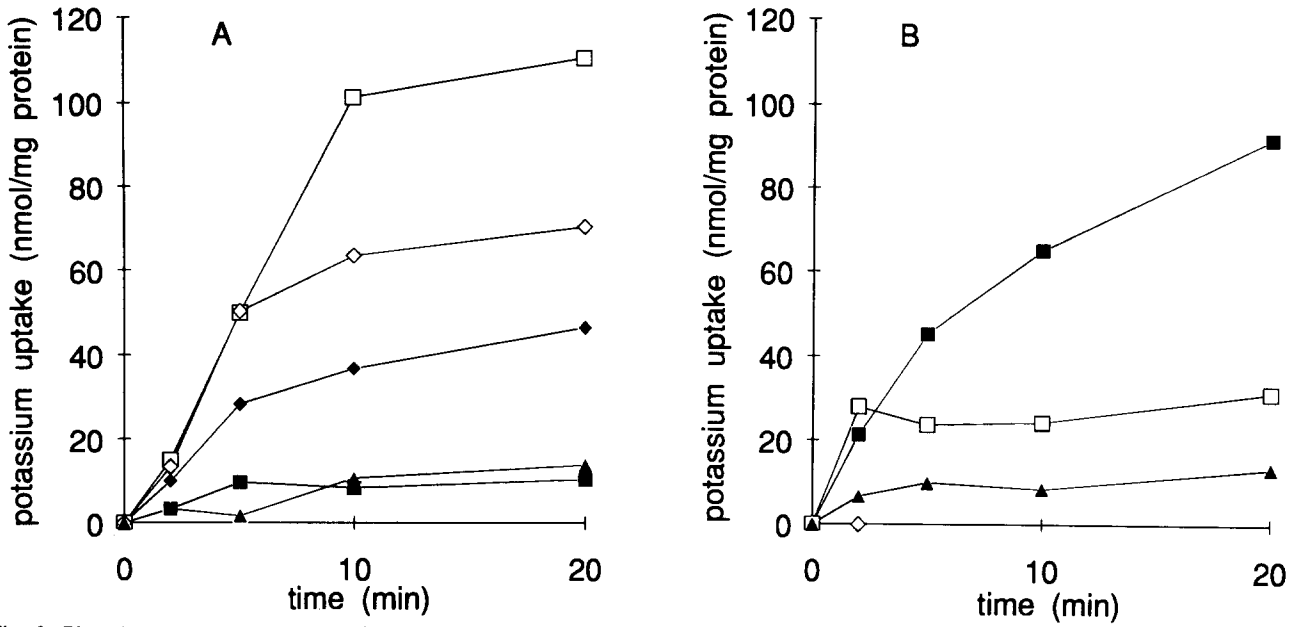

Fig. 3. Phospho*enol*pyruvate-driven K^+ uptake by right-side-out membrane vesicles. (A) Vesicles were prepared from *E. coli* TKA1000 (□——□, PEP; ■——■, no energy source; ◆——◆, PEP+bafilomycin B_1; ◇——◇, PEP+orthovanadate) and TKR2000 (▲——▲, PEP). (B) Vesicles were prepared from *E. coli* TK2240–40 (□——□, PEP; ▲——▲, D-lactate; ■——■, PEP+D-lactate). With PEP as the energy source samples were preincubated for 30 min before the reaction was started by the addition of 1 mM KCl. When D-lactate was used the preincubation time was 5 min. Orthovanadate (50 μM final concentration) and bafilomycin B_1 (100 μM final concentration) were added 10 min and 5 min, respectively, before the addition of KCl. Experimental details were otherwise as described in Materials and Methods.

transport in the presence of PEP involves the intravesicular formation of ATP and its hydrolysis by the ATP synthase with concomitant generation of a $\Delta\tilde{\mu}_{H^+}$. As expected, dicyclohexylcarbodiimide (DCCD), a potent inhibitor of the ATP synthase has essentially no effect on D-lactate-driven proline transport. In contrast, PEP-driven proline accumulation is completely abolished.

K^+ uptake via the Kdp-ATPase ist most convincingly demonstrated by membrane vesicles of TKA1000 and, to a lesser extent, of TK2240–40 in the presence of PEP (Fig. 3). In the latter case the amount of K^+ taken up could be increased by adding both, PEP and D-lactate as energy sources. K^+ transport is, however, totally absent in vesicles of TKR2000 lacking the Kdp-ATPase. That PEP does not drive proline uptake in the *unc*$^-$strains, but does fuel K^+ transport, confirms that ATP and not the proton electrochemical gradient provides the energy for K^+ transport. The uptake of K^+ in vesicles of TKA1000 was partially inhibited by orthovanadate and to a higher extent by bafilomycin B_1, both compounds being well established inhibitors of P-type ATPases [19,31].

Discussion

PEP added to the medium enters the intravesicular space via the phosphoglycerate transporter and is then used to phosphorylate ADP in a Mg^{2+}-dependent reaction catalyzed by pyruvate kinase. As shown in Fig. 1, membrane vesicles derived from strains TKA1000 (*Δunc*, *kdp*$^+$) and TKR2000 (*Δunc*, *Δkdp*) reach a steady state level of about 3.6 nmoles ATP/mg protein. Assuming an intravesicular space of 2 μl/mg protein [32] the amount of ATP synthesized is around 1.8 mM. It is interesting to note that the level of ATP in membrane vesicles of TK2240–40 can be increased if both PEP and D-lactate are present. This can be attributed to the fact that energization of the membrane by D-lactate shifts the ATP synthase from the hydrolysis towards the synthesis mode.

Subsequently, the ATP formed can be hydrolyzed by the membrane-bound ATP synthase, generating a proton electrochemical gradient that drives active transport of proline (Fig. 2). However, the ATP formed can also be used directly as an energy source to drive uptake of K^+ via the Kdp-ATPase. In membrane vesicles derived from strain TKA1000 about 110 nmol K^+/mg protein are accumulated. Assuming again an intravesicular space of 2 μl/mg protein, this would lead then to an intravesicular concentration of 55 mM K^+. Since the outside concentration of K^+ is 1 mM a concentration gradient of 55 has been established. In contrast, uptake of K^+ in membrane vesicles derived from TK2240–40 (*unc*$^+$, *kdp*$^+$) is much lower (30 nmol K^+/mg protein). This is in parallel to the observation that the level of ATP in these vesicles is roughly one third of that found in vesicles of TKA1000, due to the

presence of the ATP-hydrolyzing activity of the ATP synthase. It is worthwhile mentioning that in comparison to the uptake of K^+ (approaching 10 nmol/mg per min) the rate of proline transport is much lower (about 0.015 nmol/mg per min). This large difference in rates is mainly due to the fact that we are using 3.5 μM proline in our transport assay, which is well below the K_M for proline transport via ProP.

The partial inhibition of K^+ uptake by orthovanadate is rather surprising. This inhibitor is known to compete with phosphate for the same binding site located at the cytoplasmic surface of the Kdp-ATPase. Since an excess amount of orthovanadate has been used leading to only 36% inhibition, it is quite conceivable that some orthovanadate has reached the intravesicular space. However, it is equally well possible that some orthovanadate has entered the lumen of the vesicles via a phosphate transport system. Inhibition of K^+ uptake is much more pronounced (60%) using bafilomycin B_1. Among other bafilomycin analogs (A_1, C_1 and D), B_1 was chosen because it exhibited the strongest inhibitory effect (90% inhibition at 100 μM) on the membrane-bound Kdp-ATPase in everted vesicles. Although the binding site(s) for the macrolide antibiotic is (are) not known, the aforementioned observation, together with the hydrophobic nature of these compounds, would argue in favor of the possibility that the antibiotic exerts its effect from the cytoplasmic side of the membrane. This consideration would be in accord with the partial inhibitory effect of bafilomycin B_1 provided from the medium side of the membrane.

Finally, it is worthwhile mentioning that we have been unable to detect movement of H^+ in symport or antiport with K^+ (data not shown). This would then argue in favor of an electrogenic process for K^+ accumulation via the Kdp-ATPase.

Acknowledgements

We thank Dr. Gabriele Deckers-Hebestreit for critically reading and Johanna Petzold for typing the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 171/B5) and the Fonds der Chemischen Industrie.

References

1 Suelter, C.H. (1970) Science 168, 789–795.
2 Epstein, W. (1986) FEMS Microbiol. Rev. 39, 73–78.
3 Plack, R.H. and Rosen, B.P. (1980) J. Biol. Chem. 255, 3824–3825.
4 Booth, I.R. (1985) Microbiol. Rev. 49, 359–378.
5 Bakker, E.P., Booth, I.R., Dinnbier, U., Epstein, W. and Gajewska, A. (1987) J. Bacteriol. 169, 3743–3749.
6 Csonka, L.N. and Hanson, A.D. (1991) Annu. Rev. Microbiol. 45, 569–606.
7 Bakker, E.P. (1992) Alkali cation transport systems in prokaryotes (and articles therein), CRC Press, Boca Raton.
8 Bossemeyer, D., Schlösser, A. and Bakker, E.P. (1989) J. Bacteriol. 173, 2219–2221.
9 Dosch, D.C., Helmer, G.L., Sutton, S.H., Salvacion, F.F. and Epstein, W. (1991) J. Bacteriol. 173, 687–696.
10 Rhoads, D.B., Waters, F.B. and Epstein, W. (1976) J. Gen. Physiol. 67, 325–341.
11 Rhoads, D.B., Woo, A. and Epstein, W. (1977) Biochim. Biophys. Acta 469, 45–51.
12 Epstein, W. (1985) Curr. Top. Membr. Transport 23, 153–175.
13 Hesse, J.E., Wieczorek, L., Altendorf, K., Reicin, A.S., Dorus, E. and Epstein, W. (1984) Proc. Natl. Acad. Sci. USA 81, 4746–4750.
14 Walderhaug, M.O., Polarek, J.W., Voelkner, P., Daniel, J.M., Hesse, J.E., Altendorf, K. and Epstein, W. (1992) J. Bacteriol. 174, 2152–2159.
15 Stock, J.B., Ninfa, A.J. and Stock, A.M. (1989) Microbiol. Rev. 53, 450–490.
16 Epstein, W., Whitelaw, V. and Hesse, J. (1978) J. Biol. Chem. 253, 6666–6668.
17 Wieczorek, L. and Altendorf, K. (1979) FEBS Lett. 98, 233–236.
18 Rhoads, D.B. and Epstein, W. (1977) J. Biol. Chem. 252, 1394–1401.
19 Siebers, A. and Altendorf, K. (1988) Eur. J. Biochem. 178, 131–140.
20 Siebers, A., Wieczorek, L. and Altendorf, K. (1988) Methods Enzymol. 157, 668–680.
21 Siebers, A., Kollmann, R., Dirkes, G. and Altendorf, K. (1992) J. Biol. Chem. 267, 12717–12721.
22 Epstein, W., Walderhaug, M.O., Polarek, J.W., Hesse, J.E., Dorus, E. and Daniel, J. (1990) Phil. Trans. Royal Soc. Lond. B 326, 479–487.
23 Pedersen, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–150.
24 Polarek, J.W., Walderhaug, M.O. and Epstein, W. (1988) Methods Enzymol. 157, 655–667.
25 Hugenholtz, J., Hong, J.-S. and Kaback, H.R. (1981) Proc. Natl. Acad. Sci. USA 78, 3446–3449.
26 Epstein, W. and Davies, M. (1970) J. Bacteriol. 101, 836–843.
27 Kaback, H.R. (1971) Methods Enzymol. 22, 99–120.
28 DeLuca, M. and McElroy, W.D. (1978) Methods Enzymol. 57, 3–15.
29 Chapman, A.G., Fall, L. and Atkinson, D.E. (1971) J. Bacteriol. 108, 1072–1086.
30 Saier Jr., M.H., Wentzel, D.L., Feucht, B.U. and Judice, J.J. (1975) 250, 5089–5096.
31 Bowman, E.J., Siebers, A. and Altendorf, K. (1988) Proc. Natl. Acad. Sci. USA 85, 7972–7976.
32 Kaback, H.R. and Barnes Jr., E.M. (1971) J. Biol. Chem. 246, 5523–5531.