

## MOLECULAR PHYSIOLOGY OF THE Na<sup>+</sup>/H<sup>+</sup> ANTIPORTER IN *ESCHERICHIA COLI*

ETANA PADAN AND SHIMON SCHULDINER

*Department of Molecular and Microbial Ecology, Institute of Life Sciences, Hebrew  
University of Jerusalem, Jerusalem, Israel*

### Summary

All living cells maintain an inwardly directed Na<sup>+</sup> gradient and a constant intracellular pH. Na<sup>+</sup>/H<sup>+</sup> antiporters have been assigned an essential role in these homeostatic mechanisms in all cells. In *Escherichia coli*, two Na<sup>+</sup>/H<sup>+</sup> antiporter genes, *nhaA* and *nhaB*, have been cloned. Deletion of either one or both showed that NhaA is essential for adaptation to high salinity, for growth at alkaline pH in the presence of Na<sup>+</sup> and for challenging Li<sup>+</sup> toxicity. NhaB confers tolerance to low levels of Na<sup>+</sup> and becomes essential when the activity of NhaA limits growth.

The adaptive response to Na<sup>+</sup> is mediated by the positive regulator *nhaR*, which transduces the signal (intracellular Na<sup>+</sup>) to expression of the *nhaA* gene. We have identified Glu-134 of NhaR as part of the 'Na<sup>+</sup> sensor' of NhaA.

In agreement with the role of NhaA in pH homeostasis, its Na<sup>+</sup>-dependent expression is enhanced at alkaline pH. Reconstitution of pure NhaA and NhaB in proteoliposomes demonstrates that, whereas both are electrogenic (the H<sup>+</sup>/Na<sup>+</sup> stoichiometry of NhaA is 2), only NhaA is pH-dependent, increasing its activity 1000-fold between pH 7 and 8.5. Mutating all the histidines of NhaA shows that His-226 is part of the 'pH sensor' of NhaA.

### Introduction

Antiporters are secondary transporters, membrane proteins that couple electrochemical gradients of ions or organic solutes to drive transport reactions. In contrast to symporters, which couple reactions of the same orientation, the orientation of the driving reaction coupled by the antiporters is opposed to that of the driven reaction (Fig. 1).

Do these opposed fluxes *via* the antiporters entail a unique mechanism, different from that of the symporters? We cannot answer this question since, as yet, we do not know the mechanism of either transport system. However, since the antiporters couple the fluxes of cytoplasmic or organellar metabolites to those of important ions, their biology shows certain unique properties. Some of them are directly coupled to a sophisticated cascade of reactions, including scalar reactions, that occur in the cytoplasm (Maloney, 1992; Poolman and Konings, 1993). A case in point is the OxlT system, which exchanges oxalate with formate and is coupled to oxalate decarboxylase, so that the overall reactions also yield net H<sup>+</sup> pumping (Maloney, 1992).

Furthermore, the role of the antiporters is not only in the transport of solutes into the

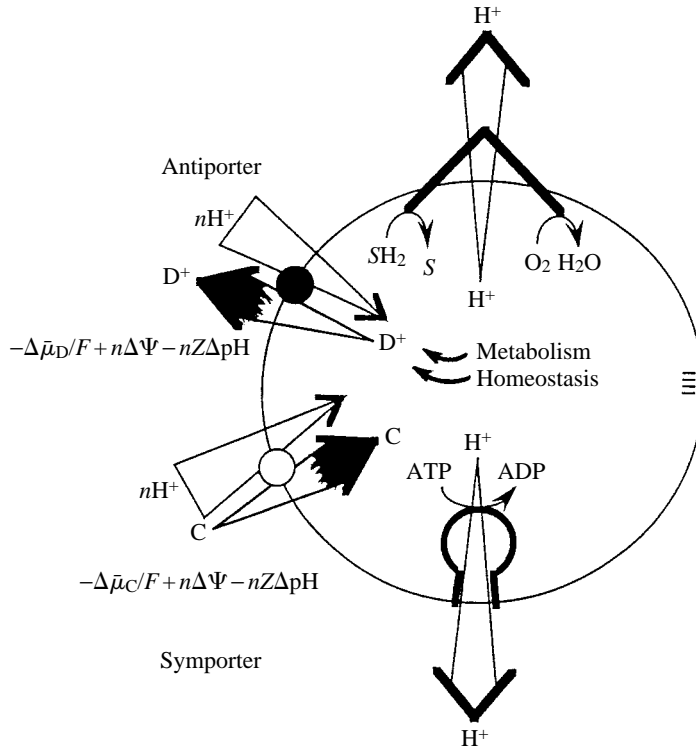


Fig. 1. Antiporter *versus* symporter. The driving forces on the respective solutes (D or C) are shown;  $F$ , Faraday constant;  $Z=2.3RT/F$ ;  $\Delta\Psi$ , potential difference;  $\Delta\mu$ , electrochemical potential difference;  $\Delta\text{pH}$ , pH difference;  $S$ , substrate.

cells or organelles, but also, or even primarily, in the very basic mechanisms of homeostasis of ions in cells. Examples are the  $\text{Na}^+/\text{H}^+$  (Padan and Schuldiner, 1992, 1993, 1994; Schuldiner and Padan, 1992; Krulwich *et al.* 1988, 1990),  $\text{K}^+/\text{H}^+$ ,  $\text{Ca}^{2+}/\text{H}^+$  (Rosen, 1986; Poolman and Konings, 1993),  $\text{Ca}^{2+}/\text{Na}^+$  (Nicoll *et al.* 1990) and  $\text{Cl}^-/\text{bicarbonate}$  antiporters (Olsnes *et al.* 1987; Raley-Susman *et al.* 1991). It is therefore not surprising that the regulation of both the activity and/or expression of certain antiporters is known to be very sophisticated, involving sensors to the specific ionic signals and signal transduction pathways (Aronson, 1985; Raley-Susman *et al.* 1991; Padan and Schuldiner, 1993, 1994; Sardet *et al.* 1990, 1991a,b; Grinstein *et al.* 1992).

The  $\text{Na}^+/\text{H}^+$  antiporters have been studied extensively. They exchange  $\text{Na}^+$  for  $\text{H}^+$ , ubiquitous ions that are involved in cell energetics and numerous cellular processes. Indeed,  $\text{Na}^+/\text{H}^+$  antiporters have been assigned vital and general roles in all living cells. In most bacteria and plant cells, they are involved in the pH homeostasis of the cytoplasm, as well as in the expulsion of  $\text{Na}^+$  which, at high concentrations, is toxic to the cytoplasm. In addition,  $\text{Na}^+$  excretion is essential in bacteria for maintaining an inwardly directed  $\text{Na}^+$  gradient, which serves as a driving force for many transport systems. In animal cells, the  $\text{Na}^+/\text{H}^+$  antiporters are essential for pH homeostasis and for the regulation of cell volume.

In accordance with their vital roles, the Na<sup>+</sup>/H<sup>+</sup> antiporters have been found in the cytoplasmic membrane of all living cells from microorganisms to plant and animal cells (Padan and Schuldiner, 1993, 1994). Only one bacterium, *Clostridium feruidus*, that appears to lack this antiporter activity is known (Speelmans *et al.* 1993). They have also been found in membranes of various intracellular organelles, in mitochondria, in plant vacuoles and in the storage granules of animal cells.

The broad occurrence and major multifactorial functions of antiporters in various organisms prompts the questions of the differences between the antiporters, and the pattern of their activity and their regulation in the various organisms (Padan and Schuldiner, 1993). How they are involved, at the same time, in the circulation of H<sup>+</sup> and Na<sup>+</sup> in cells is another interesting question on which we will focus in this review through analysis of the antiporters of *Escherichia coli*, with special emphasis on the Na<sup>+</sup> and H<sup>+</sup> sensors.

### The Na<sup>+</sup>/H<sup>+</sup> antiporter genes and proteins of *Escherichia coli*

*Escherichia coli* has two specific Na<sup>+</sup>/H<sup>+</sup> antiporters systems – *nhaA* (Goldberg *et al.* 1987; Karpel *et al.* 1988) and *nhaB* (Pinner *et al.* 1992). The respective genes have been cloned and mapped (Fig. 2) and their nucleotide sequences determined. The deduced amino acid sequences suggest that both proteins have extensive helical structures repetitively spanning the membrane, as to be expected for transport proteins (Fig. 3). When either of the genes is present at a high copy number in cells (after introduction on multicopy plasmids), the Na<sup>+</sup>/H<sup>+</sup> antiporter activity, but no other antiporter activities (K<sup>+</sup>/H<sup>+</sup>, Ca<sup>2+</sup>/H<sup>+</sup>), increases dramatically (Goldberg *et al.* 1987; Karpel *et al.* 1988; Pinner *et al.* 1992). This increase can be observed using the Acridine Orange technique, which measures changes in steady-state pH differences across the membrane caused by Na<sup>+</sup> addition, or by directly monitoring <sup>22</sup>Na fluxes in isolated membrane vesicles.

The NhaA protein has been purified in a functional form (Taglicht *et al.* 1991). This endeavour involved the identification of the protein, over-expressing it, developing solubilization and purification procedures, and finally reconstituting the purified protein in proteoliposomes to test its activity. This achievement was crucial in order to prove that *nhaA* is a structural gene and that its product, a single polypeptide, is indeed an antiporter. Furthermore, since this protein is the first pure active Na<sup>+</sup>/H<sup>+</sup> antiporter to be isolated, it allowed us to study aspects of the antiporter mechanism that could not easily or unequivocally be studied in intact cells or membrane vesicles.

The antiporter Na<sup>+</sup>:H<sup>+</sup> stoichiometry is a long-debated issue. With the purified system, we have recently proved that NhaA is electrogenic and that its stoichiometry is 2H<sup>+</sup> for every Na<sup>+</sup> (Taglicht *et al.* 1993).

Another aspect, which is related to the role of the antiporter in pH homeostasis, is the effect of pH on its activity. Working with isolated membrane vesicles, Leblanc and his colleagues suggested that the Na<sup>+</sup>/H<sup>+</sup> antiporter activity is highly sensitive to pH (Bassilana *et al.* 1984). Our results with purified NhaA show that this antiporter accounts for the pH-sensitivity observed in membrane vesicles (Taglicht *et al.* 1991). The purified NhaA antiporter is highly sensitive to pH, changing its activity by three orders of magnitude over the pH range 7–8.5 (Taglicht *et al.* 1991; Fig. 4). These results imply that

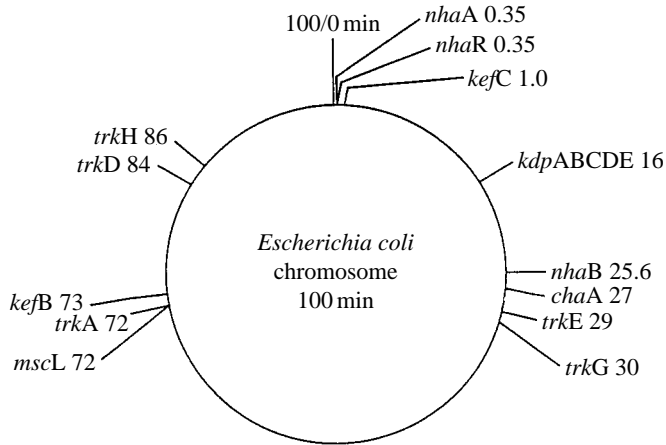


Fig. 2. Genes involved in transport of the major cations in *Escherichia coli*. *kdp*, *trk*, *kef* and *mscL* (Bakker, 1988; Sukharev *et al.* 1994) are  $K^+$  transport loci. *nhaA*, *nhaB* and *nhaR* are  $Na^+$  transport loci (Goldberg *et al.* 1987; Rahav-Manor *et al.* 1992; Pinner *et al.* 1992). *chaA* is a  $Ca^{2+}$  transport locus (Ivey *et al.* 1993).

NhaA functions simultaneously both as a pH sensor and as a pH titrator. Identification of the 'pH sensor' of this protein has since become an intriguing challenge (Gerchman *et al.* 1993).

Recently, we have purified NhaB and reconstituted it in proteoliposomes in a functional form. Like NhaA, NhaB is electrogenic (E. Pinner, E. Padan and S. Schuldiner, unpublished results). However, it differs from NhaA in three aspects. (1) Its ion specificity is different, having a higher affinity for  $Na^+$ , but a lower affinity for  $Li^+$  than those of NhaA (Schuldiner and Padan, 1992). (2) NhaB is sensitive to amiloride, an inhibitor of the eukaryotic antiporter, whereas NhaA is insensitive (E. Pinner, E. Padan and S. Schuldiner, unpublished results). (3) Whereas the activity of NhaA is dramatically affected by pH, NhaB is almost indifferent to it (Schuldiner and Padan, 1993).

### Physiology of the $Na^+/H^+$ antiporters

It is apparent that two very different antiporters exist in the cytoplasmic membrane of *E. coli*, raising questions about their different roles and whether additional antiporters participate in the  $Na^+/H^+$  antiporter activity of the cells. In order to answer these questions, *E. coli* strains with the *nhaA* gene (Padan *et al.* 1989) or the *nhaB* gene (Pinner *et al.* 1993) or both *nhaA* and *nhaB* deleted were constructed. A comparison of the various mutants with the wild type allowed us to deduce the role of the various antiporters.

The wild-type antiporter is sensitive to  $Li^+$ , but the mutant  $\Delta nhaA$  is much more

Fig. 3. Model of the secondary structure of NhaA and NhaB. The models shown are based on the predictions of the hydropathic profiles (Karpel *et al.* 1988; Pinner *et al.* 1992). The arrow highlights His-226.

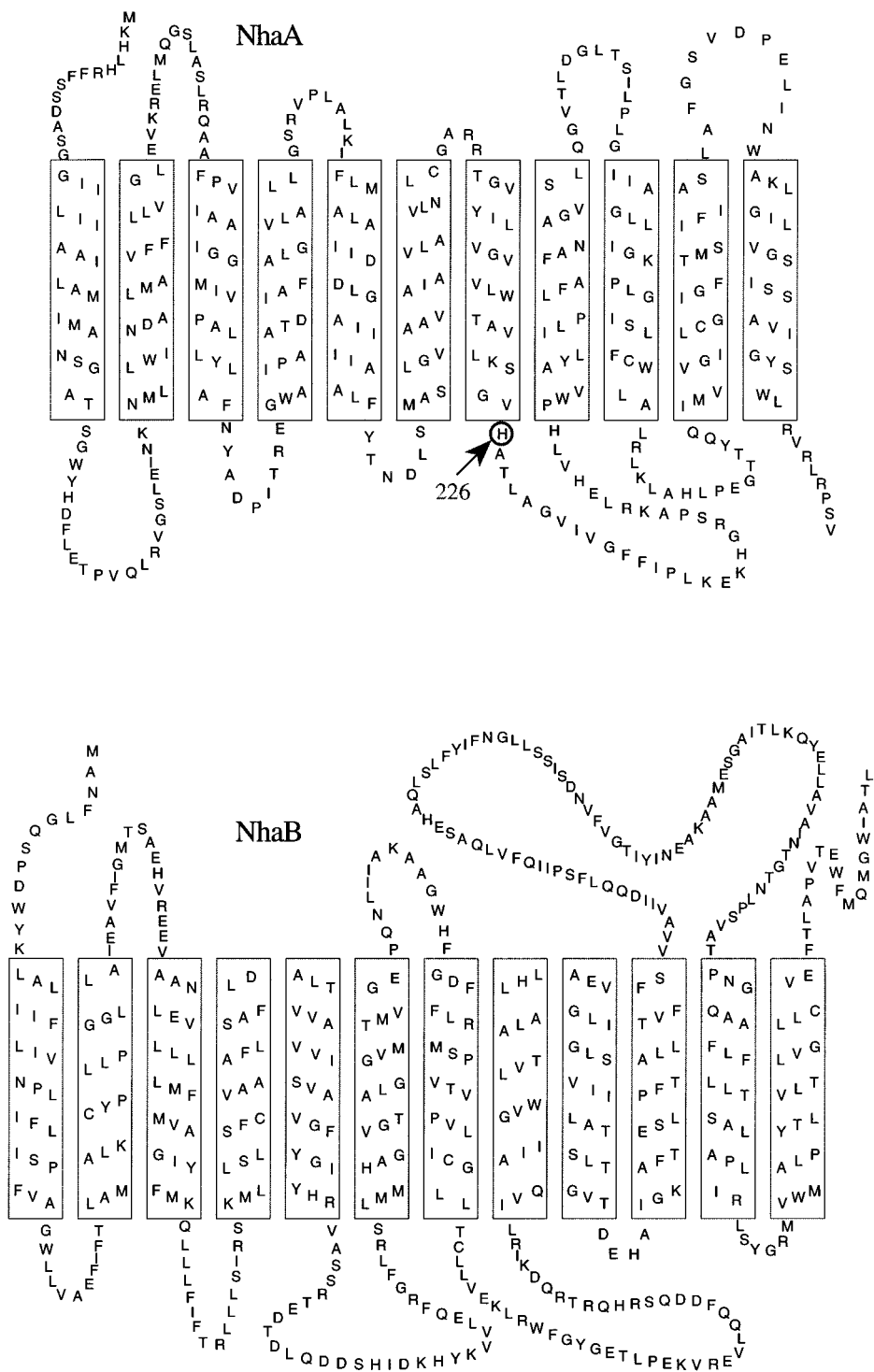


Fig. 3

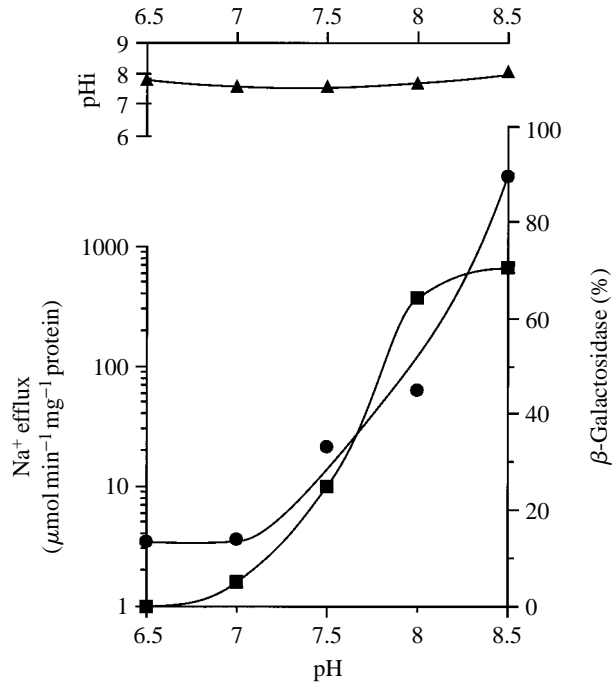


Fig. 4. pH-dependence of NhaA activity and *nhaA* expression. The data for Na<sup>+</sup> efflux (■) were obtained with purified NhaA reconstituted in proteoliposomes (Taglicht *et al.* 1991). The data for *nhaA* expression (●) were obtained with *nhaA-lacZ* protein fusion (Karpel *et al.* 1991). Intracellular pH (pHi) of *E. coli* versus extracellular pH (Padan *et al.* 1976) is also shown (▲).

sensitive to the ion; 100 mmol l<sup>-1</sup> LiCl completely arrests its growth (Padan *et al.* 1989). The  $\Delta nhaA$  mutant is sensitive to Na<sup>+</sup>, in contrast to the wild type (Padan *et al.* 1989). At pH 7.5, the wild type grows in the presence of up to 0.7 mol l<sup>-1</sup> Na<sup>+</sup>, but the mutant is drastically inhibited by 0.4 mol l<sup>-1</sup> Na<sup>+</sup>. This inhibition is not due to an osmotic effect, since 0.7 mol l<sup>-1</sup> KCl had no effect on  $\Delta nhaA$ . Instead, this inhibition is attributed to the lack of the *nhaA* gene since, upon transformation of  $\Delta nhaA$  with multicopy plasmid bearing *nhaA*, the transformants become as Na<sup>+</sup>-resistant as the wild type. One can therefore deduce that *nhaA* is essential for the adaptation of *E. coli* to high salinity.

As long as Na<sup>+</sup> is withheld from the medium,  $\Delta nhaA$  grows at alkaline pH up to pH 8.6, as the wild type does, implying that *nhaA* is not required for pH homeostasis under these conditions. However, an alkaline pH markedly increases the sensitivity of  $\Delta nhaA$  to Na<sup>+</sup>. At a pH of 8.6, even 100 mmol l<sup>-1</sup> NaCl is inhibitory to the mutant (Padan *et al.* 1989). We therefore concluded that *nhaA* is indispensable for growth at alkaline pH in the presence of Na<sup>+</sup>.

The mutant deleted of *nhaB* shows no sign of a Na<sup>+</sup>-sensitive phenotype, implying that, as long as *nhaA* is present, it can by itself cope with Na<sup>+</sup>, at both neutral and alkaline pH (Pinner *et al.* 1993). However, the double mutant, lacking both  $\Delta nhaA$  and  $\Delta nhaB$ , is more sensitive to Na<sup>+</sup> than is  $\Delta nhaA$  alone. Although  $\Delta nhaA$  grows at pH 7.5 up to

400 mmol l<sup>-1</sup> NaCl,  $\Delta nhaA\Delta nhaB$  ceases to grow at 30–50 mmol l<sup>-1</sup> NaCl, even at neutral pH. It is therefore concluded that, in the absence of *nhaA*, *nhaB* can confer some Na<sup>+</sup>-resistance on the cells, but its resistance is much lower than that of *nhaA*. At neutral pH, *nhaB* allows growth up to 300–400 mmol l<sup>-1</sup> NaCl, whereas *nhaA* supports growth up to 700 mmol l<sup>-1</sup>. At alkaline pH, its capacity is even lower, allowing growth only up to 100 mmol l<sup>-1</sup>.

Isolated membrane vesicles derived from the double mutant  $\Delta nhaA\Delta nhaB$  revealed no Na<sup>+</sup>/H<sup>+</sup> antiporter activity (Pinner *et al.* 1993). It can therefore be concluded that NhaA and NhaB are the only specific Na<sup>+</sup>/H<sup>+</sup> antiporters in the membrane of *E. coli*, except for the possible existence of silent antiporters.

### Regulation of transcription of *nhaA*, the ‘Na sensor’

Understanding how the expression of *nhaA* is regulated is crucial for an understanding of the pattern of operation of the two antiporters in the cell. For this purpose, a strain containing *nhaA* fused in frame to a reporter gene, *lacZ*, was constructed (Karpel *et al.* 1991). Hence, by measuring  $\beta$ -galactosidase activity under different conditions, the signals affecting the expression of *nhaA* were identified (100 mmol l<sup>-1</sup> Na<sup>+</sup> or Li<sup>+</sup> at pH 7.5). This effect is specific to these cations, since neither K<sup>+</sup> nor glutamate has any effect. pH alone does not activate the system. However, it markedly potentiated the sensitivity of the expression system to Na<sup>+</sup>. Whereas 7 mmol l<sup>-1</sup> Na<sup>+</sup> has no effect up to pH 7.5, at pH 8.5 this Na<sup>+</sup> concentration increases expression sevenfold (Fig. 4).

It is apparent that the pattern of regulation of *nhaA* reflects its role in adaptation to high salinity and alkaline pH; thus, *nhaA* is expressed under conditions of high Li<sup>+</sup> or Na<sup>+</sup> concentration and at alkaline pH (in the presence of Na<sup>+</sup>).

The inducibility of *nhaA* implies that a regulatory protein or proteins must exist for *nhaA*. Downstream of *nhaA*, there is an open reading frame which we call *nhaR*. Deletion of *nhaR* from the chromosome yields Li<sup>+</sup>- and Na<sup>+</sup>-sensitive cells even when they have *nhaA* (Rahav-Manor *et al.* 1992). Furthermore, multicopy *nhaR* increases the expression of the *nhaA-lacZ* in the fusion-bearing strain, and this increase is completely Na<sup>+</sup>-dependent. Taken together, these results indicate that *nhaR* is a positive regulator of *nhaA*, which works in the *trans* position, in a Na<sup>+</sup>-dependent fashion.

If it is the positive regulator for *nhaA* expression, NhaR should bind to the regulatory signals of *nhaA* and promote transcription. Indeed, Northern analysis shows that Na<sup>+</sup> induces *nhaA* transcription in a manner dependent on *nhaR* (Carmel *et al.* 1994), and partially purified NhaR specifically binds the *nhaA* promoter region, as shown in a gel retardation system (Rahav-Manor *et al.* 1992).

On the basis of protein homology (41 % similarity), NhaR belongs to a large family of positive regulatory proteins called the LysR family, all members of which contain in their N termini a helix-turn-helix motif that is believed to bind DNA (Henikoff *et al.* 1988). Most importantly, several of these proteins are part of a signal transduction pathway involved in the response to environmental stress (Storz *et al.* 1990). We suggest that NhaR is part of a signal transduction system that is essential for coping with Na<sup>+</sup> and pH stress.

Changes in extracellular  $\text{Na}^+$  concentration must be sensed by NhaR, since the effect of NhaR on *nhaA* expression is  $\text{Na}^+$ -dependent. It is conceivable that a change in intracellular  $\text{Na}^+$  concentration, which accompanies the change in the extracellular concentration of the ion, serves as the immediate signal for the NhaR-dependent expression of *nhaA*. If intracellular  $\text{Na}^+$  is indeed the immediate signal, then any event which decreases or increases intracellular  $\text{Na}^+$  concentration should change the expression. Transformation of the fusion-bearing strain with multicopy plasmids bearing either *nhaA* or *nhaB* inhibits the induction of *nhaA* caused by  $\text{Na}^+$ . Since these genes share very little homology but a common activity –  $\text{Na}^+$  extrusion – we suggest that an increase in the intracellular  $\text{Na}^+$  concentration serves as the immediate on-signal for NhaR-dependent *nhaA* expression (Padan and Schuldiner, 1992). Accordingly, *nhaA-lacZ* is fully expressed at a very low  $\text{Na}^+$  concentration ( $10\text{mmol l}^{-1}$ ) in a  $\Delta\text{nhaA}\Delta\text{nhaB}$  mutant (E. Pinner, E. Padan and S. Schuldiner, unpublished results).

Is the effect of intracellular  $\text{Na}^+$  on NhaR direct? One approach to answering this question which we have already undertaken is biochemical: we studied the effect of  $\text{Na}^+$  on the interaction of NhaR with the DNA and on *nhaA* transcription *in vitro*.

Another approach is genetic. Since the induction of *nhaA* by  $\text{Na}^+$  occurs *via* NhaR, it is conceivable that a ‘ $\text{Na}^+$  sensor’ site on NhaR can be identified by mutations affecting the  $\text{Na}^+$ -sensitivity of the expression system. Should such a mutation increase the affinity for  $\text{Na}^+$  of the expression system, then, at a given  $\text{Na}^+$  concentration, it might even increase the  $\text{Na}^+/\text{H}^+$  antiporter activity in the membrane above that in the wild type. A previously isolated mutation, designated *antup*, was found to increase the  $\text{Na}^+/\text{H}^+$  antiporter activity, thereby conferring  $\text{Li}^+$ -resistance upon wild-type cells which are otherwise  $\text{Li}^+$ -sensitive (Nha<sup>up</sup> phenotype) (Niiya *et al.* 1982; Goldberg *et al.* 1987). We recently found that the Nha<sup>up</sup> mutation resides in the C-terminus of NhaR and is a Glu-134→Ala-134 substitution in the protein (Carmel *et al.* 1994). This mutation increases the affinity for  $\text{Na}^+$  of *nhaR*-mediated *nhaA* transcription and expression. It is therefore compelling to speculate that Glu-134 is part of the ‘ $\text{Na}^+$  sensor’ of NhaR.

### Regulation of NhaA activity: the ‘pH sensor’

There is a strong pH homeostasis in *E. coli*, as in other bacteria (Padan *et al.* 1976, 1981, 1989; Padan and Schuldiner, 1986, 1987; Slonczewski *et al.* 1982; Krulwich *et al.* 1988, 1990), and the  $\text{Na}^+/\text{H}^+$  antiporters have been assigned an essential role in this homeostasis. It is therefore surprising that  $\Delta\text{nhaA}\Delta\text{nhaB}$  grows like the wild type as long as  $\text{Na}^+$  is withheld. Hence, if pH homeostasis still exists in this mutant, another system must be responsible for it.

Interestingly, the tolerance to  $\text{Na}^+$  is linked very closely to pH. The sensitivity of  $\Delta\text{nhaA}$  to  $\text{Na}^+$  increases dramatically with increasing pH, implying that the dependence of growth on *nhaA* increases at alkaline pH. Accordingly, the inducibility of *nhaA* increases with increasing pH and its physiological importance is more pronounced at alkaline pH.

There are two possible explanations for the increasing physiological importance of *nhaA* at increased pH observed with  $\Delta\text{nhaA}$ . The first alternative is that pH homeostasis



is not required at alkaline pH and that *nhaA* is needed only to alleviate the Na<sup>+</sup> toxicity which increases with pH because of the interchangeability of Na<sup>+</sup> and H<sup>+</sup> shown in various systems (for a review, see Padan and Schuldiner, 1992). The other alternative is that *nhaA* is involved in the regulation of both pH and [Na<sup>+</sup>] and therefore the pH homeostasis that is essential at all pH values is hampered as Na<sup>+</sup> load is increased. In both cases, NhaA must sense pH and its activity must increase with pH.

As mentioned above (Fig. 4 and Taglicht *et al.* 1991), the activity of purified NhaA is markedly dependent on pH, implying that NhaA is equipped with a 'pH sensor'. It is anticipated that a residue that senses pH has an ionizable H<sup>+</sup> with a pK in the sensing range. A very good candidate is histidine, which has a pK of around 6.0. A histidine-specific modifier, diethyl pyrocarbonate (DEPC), inhibits Na<sup>+</sup>/H<sup>+</sup> antiporter activity in isolated membrane vesicles (Damiano *et al.* 1985) and in proteoliposomes reconstituted with purified NhaA (Taglicht *et al.* 1993). Furthermore, His-322 of the Lac permease has been implicated in coupling the transport of lactose to the proton gradient (Padan *et al.* 1985; Kaback, 1988).

NhaA has eight histidines, each of which has been altered by site-directed mutagenesis to arginine (Gerchman *et al.* 1993). Seven of the mutants behaved exactly like the wild type, both at neutral and at alkaline pH. However, the substitution of His-226 to arginine dramatically affected the phenotype.

At pH 7.5, Arg-226 grows like the wild type at all Na<sup>+</sup> concentrations, whereas at pH 8.5, the growth of Arg-226 in the presence of 0.1 mol l<sup>-1</sup> Na<sup>+</sup> is completely arrested. The expression of NhaA is not affected by the mutation, nor is its maximal activity. However, the pH-sensitivity is markedly affected, as observed in isolated membrane vesicles. At acidic pH, Arg-226 is even more active than the wild type, whereas at alkaline pH, Arg-226 is inhibited. Thus, the pH profile of Arg-226 is shifted by half a pH unit towards the acidic range.

We have recently changed this His-226 to aspartate (H226R) and found that the pH profile of NhaA is shifted by about half a pH unit towards the basic range. It is therefore suggested that His-226 resides in the pH-sensing domain of NhaA. Revertants and second-site revertants of Arg-226 further substantiate the importance of His-226. They were selected after chemical mutagenesis of the plasmid bearing the H226R mutation, transformation into the  $\Delta nhaA\Delta nhaB$  strain and growth of the transformants at alkaline pH in the presence of Na<sup>+</sup>. These conditions are non-permissive unless H226R is reversed to His-226 or second-site reversion occurs. Of the 37 independent isolates obtained, 23 were true revertants to His-226 and the rest were second-site revertants, which should enable further identification of the pH-sensing domain (A. Rimon, Y. Gerchman, S. Schuldiner and E. Padan, unpublished results).

### **Cloning of new antiporters in search for common denominators among the antiporters**

The Na<sup>+</sup>/H<sup>+</sup> antiporters are widely distributed and are involved in homeostasis of pH and/or Na<sup>+</sup> in all cells. The human antiporter, NHE-1, also has a sensor but in this case it

functions as a mirror image of NhaA, reflecting the different pH ranges challenging the respective cells (Padan and Schuldiner, 1994). It would be most intriguing to identify the amino acids residues in this sensor.

Can we find common molecular denominators in the  $\text{Na}^+/\text{H}^+$  antiporter family? Interestingly, there is a sequence conservation among the Nhe family (Tse *et al.* 1993), but there is very little homology between it and NhaA, and even between NhaA and NhaB (Padan and Schuldiner, 1994). Do these results suggest that there are no universal  $\text{Na}^+$  and  $\text{H}^+$  recognition and exchange sites and that, instead, different sequences accomplish similar functions? Another possibility is that very few conserved residues are adequate to carry out these functions, even when they are dispersed throughout the protein. Identification of the domains necessary for activity regulation and  $\text{H}^+$ - and  $\text{Na}^+$ -sensing is certainly one of the future challenges.

Cloning of novel antiporter genes is required to identify conserved sequences in this family of proteins. Two strategies for cloning of  $\text{Na}^+/\text{H}^+$  antiporter genes have been advanced, each based on one of the two substrates,  $\text{Li}^+$  and  $\text{Na}^+$ , of all  $\text{Na}^+/\text{H}^+$  antiporters (Padan and Schuldiner, 1994). On a concentration basis,  $\text{Li}^+$  is 10 times more toxic than  $\text{Na}^+$ , both to wild-type *E. coli* (Goldberg *et al.* 1987) and to fission yeast (Jia *et al.* 1992). Therefore,  $\text{Li}^+$  provides a screen for cells capable of maintaining low internal  $\text{Na}^+$  or  $\text{Li}^+$  levels without selecting for osmotolerance. Another advantage of  $\text{Li}^+$  selection over that for  $\text{Na}^+$  is that it can be applied directly to wild-type cells. Realizing this advantage of  $\text{Li}^+$  selection, Niiya *et al.* (1982) isolated a mutant *nhaA*<sup>up</sup> that confers  $\text{Li}^+$ -resistance and increases the  $\text{Na}^+/\text{H}^+$  antiporter activity of *E. coli*. As described above, this mutation is an E134A substitution in NhaR, the positive regulator of *nhaA*, which increases  $\text{Na}^+/\text{H}^+$  activity by increasing the affinity for  $\text{Na}^+$  of the NhaR system (Carmel *et al.* 1994). Mapping this mutation, utilizing  $\text{Li}^+$  selection and assuming that the multicopy antiporter gene increases antiporter activity, we cloned *nhaA* out of a multicopy plasmid DNA library containing sequences overlapping the *nhaA* locus (Goldberg *et al.* 1987). Using a similar approach, *sod2* antiporter has been cloned from *Schizosaccharomyces pombe* (Jia *et al.* 1992).

Although wild-type *E. coli* cells transformed with multicopy plasmids bearing *nhaA* become  $\text{Li}^+$ -resistant compared with the wild type, their tolerance to  $\text{Na}^+$  is unchanged, implying that other factors, such as adaptation to increased osmolarity, determine the upper level of resistance to  $\text{Na}^+$ . This result also shows that, in contrast to wild-type cells mutants,  $\Delta nhaA$  or  $\Delta nhaA\Delta nhaB$ , which are  $\text{Na}^+$ -sensitive because of the lack of the antiporters, are most suitable for applying  $\text{Na}^+$  selection and cloning by complementation DNA inserts encoding  $\text{Na}^+/\text{H}^+$  antiporter genes (Fig. 5). Using this approach and the *E. coli* mutants, various antiporter genes have been cloned from very different bacteria, including *nhaB* from *E. coli* (Pinner *et al.* 1992) and *nhaC* from an alkaliphile *Bacillus firmus* OF4 (Ivey *et al.* 1991). A similar approach applied to *Enterococcus hirae* yielded *napA* antiporter (Waser *et al.* 1992).

#### Other systems conferring $\text{Na}^+$ resistance in *Escherichia coli*

The  $\text{Na}^+$ -sensitivity revealed in the antiporter mutants ( $\Delta nhaA$  or  $\Delta nhaA\Delta nhaB$ ) is

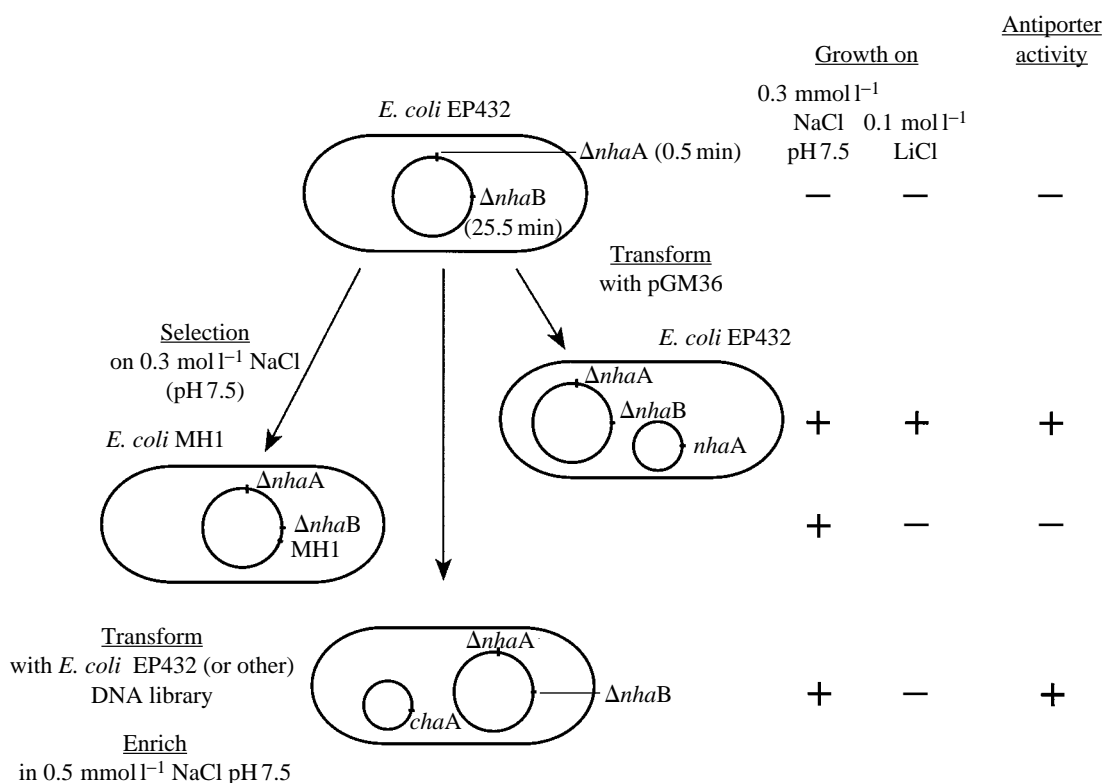


Fig. 5. The use of *Escherichia coli* strain EP432 ( $\Delta nhaA\Delta nhaB$ ) for the identification and cloning of genes involved in  $Na^+$  metabolism. The general strategy is outlined on the left-hand side of the figure. EP432 is a  $\Delta nhaA\Delta nhaB$  strain (Pinner *et al.* 1993). pGM36 is a pBR322 derivative bearing *nhaA* (Karpel *et al.* 1988); MH1 is a second-site revertant of  $\Delta nhaA\Delta nhaB$  (M. Harel, Y. Olami, P. Dibrov, S. Schuldiner and E. Padan, unpublished results). *chaA* is a putative  $Ca^{2+}/H^+$  antiporter of *E. coli*, which complements  $\Delta nhaA\Delta nhaB$  (Ivey *et al.* 1993). The respective phenotypes are shown on the right-hand side of the figure.

consistent with the role assigned to the antiporters in  $Na^+$  excretion from bacterial cells (Padan and Schuldiner, 1992). It can be anticipated that, unless other  $Na^+$ -expelling machinery exists, in the absence of the antiporters  $Na^+$  will accumulate in the cells to growth-inhibitory levels. Primary  $Na^+$  pumps were purified from various bacteria and have been shown to be involved in  $Na^+$  excretion (Dimroth, 1987, 1992a,b; Tokuda, 1992; Tokuda and Unemoto, 1985). Furthermore, on the basis of studies with intact cells (Dibrov, 1991; Skulachev, 1984, 1988) and isolated membrane vesicles (Avetisyan *et al.* 1989, 1993), the existence of primary  $Na^+$  pumps has been implicated in *E. coli*. However, the presence of the  $Na^+/H^+$  antiporters in these preparations complicates the interpretation of these results.

Since the  $\Delta nhaA\Delta nhaB$  mutant does not have the specific  $Na^+/H^+$  antiporters, it affords two approaches in a search for the existence of  $Na^+$ -excreting machinery, other than the  $Na^+/H^+$  antiporters (Fig. 5). Given that there exist antiporter systems or pumps

with very low affinity for  $\text{Na}^+$  and/or  $V_{\max}$ , amplification of these systems on multicopy plasmid may confer some  $\text{Na}^+$ -resistance to  $\Delta nhaA\Delta nhaB$ . Indeed, using this approach, *chaA* has been cloned (Ivey *et al.* 1993). On the basis of its homology to calsequestrin and its promotion of  $\text{Ca}^{2+}/\text{H}^+$  exchange activity in membranes, *chaA* appears to be a  $\text{Ca}^{2+}/\text{H}^+$  antiporter with a low affinity for  $\text{Na}^+$ .

The  $\text{Na}^+$ -sensitivity of the  $\Delta nhaA\Delta nhaB$  strain provides yet another approach for the identification of systems conferring  $\text{Na}^+$ -resistance (Fig. 5). It affords a powerful selection (growth on  $\text{Na}^+$ ) of second-site revertants resistant to  $\text{Na}^+$ . Using this approach, we have recently isolated MH1, a second-site revertant of  $\Delta nhaA\Delta nhaB$ . It bears a mutation at around 27 min on the *E. coli* chromosome, which confers  $\text{Na}^+$ - but not  $\text{Li}^+$ -resistance and partially restores the  $\text{Na}^+$  excretion capacity in the absence of the  $\text{Na}^+/\text{H}^+$  antiporters (M. Harel, P. Dibrov, Y. Olami, E. Pinner, S. Schuldiner and E. Padan, unpublished results).

The research in the authors' laboratory was supported by grants from the US–Israel Binational Science Foundation and the Basic Research Foundation of the Israel Academy of Sciences and Humanities.

### References

- ARONSON, P. S. (1985). Kinetic properties of the plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger. *A. Rev. Physiol.* **47**, 545–560.
- AVETISYAN, A. V., BOGACHEV, A. V., MURTASINA, R. A. AND SKULACHEV, V. P. (1993). ATP-driven  $\text{Na}^+$  transport and  $\text{Na}^+$ -dependent ATP synthesis in *Escherichia coli* grown at low  $\Delta\mu_{\text{H}^+}$ . *FEBS Lett.* **3**, 267–270.
- AVETISYAN, A. V., DIBROV, P. A., SKULACHEV, V. P. AND SOKOLOV, M. V. (1989). The  $\text{Na}^+$ -motive respiration in *Escherichia coli*. *FEBS Lett.* **254**, 17–21.
- BAKKER, E. P. (1988). Control of futile transmembrane potassium cycling in *Escherichia coli*. In *The Roots of Modern Biochemistry* (H. Kleinkauf, H. von Dohren and L. Jaenicke, eds), pp. 699–705. Berlin, New York: Walter de Gruyter and Co.
- BASSILANA, M., DAMIANO, E. AND LEBLANC, G. (1984). Kinetic properties of  $\text{Na}^+$  antiport in *Escherichia coli* membrane vesicles. Effects of imposed electrical potential, proton gradient and internal pH. *Biochemistry, N.Y.* **23**, 5288–5294.
- CARMEL, O., DOVER, N., RAHAV-MANOR, O., DIBROV, P., KIRSCH, D., KARPEL, R., SCHULDINER, S. AND PADAN, E. (1994). A single amino acid substitution (glu134→ala134) in *nhaR1* increases the inducibility by  $\text{Na}^+$  of *nhaA*, a  $\text{Na}^+/\text{H}^+$  antiporter gene in *Escherichia coli*. *EMBO J.* (in press).
- DAMIANO, E., BASSILANA, M. AND LEBLANC, G. (1985). Chemical modifications of the  $\text{Na}^+/\text{H}^+$  antiport in *Escherichia coli* membrane vesicles. *Eur. J. Biochem.* **148**, 183–188.
- DIBROV, P. A. (1991). The role of sodium ion transport in *Escherichia coli* energetics. *Biochim. biophys. Acta* **1056**, 209–224.
- DIMROTH, P. (1987). Sodium ion transport: Decarboxylases and other aspects of sodium ion cycling in bacteria. *Microbiol. Rev.* **51**, 320–340.
- DIMROTH, P. (1992a).  $\text{Na}^+$ -extrusion coupled to decarboxylation. In *Alkali Cation Transport Systems in Prokaryotes* (ed. E. Bakker), pp. 77–100. Boca Raton, FL: CRC Press.
- DIMROTH, P. (1992b). The  $\text{Na}^+$ -translocating ATP-synthase from *Propionigenium modestum*. In *Alkali Cation Transport Systems in Prokaryotes* (ed. E. Bakker), pp. 139–154. Boca Raton, FL: CRC Press.
- GERCHMAN, Y., OLAMI, Y., RIMON, A., TAGLICHT, D., SCHULDINER, S. AND PADAN, E. (1993). Histidine-226 is part of the pH sensor of *NhaA*, a  $\text{Na}^+/\text{H}^+$  antiporter in *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1212–1216.
- GOLDBERG, B. G., ARBEL, T., CHEN, J., KARPEL, R., MACKIE, G. A., SCHULDINER, S. AND PADAN, E.

- (1987). Characterization of Na<sup>+</sup>/H<sup>+</sup> antiporter gene of *E. coli*. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2615–2619.
- GRINSTEIN, S., WOODSIDE, M., SARDET, C., POUYSSEUR, J. AND ROTIN, D. (1992). Activation of the Na<sup>+</sup>/H<sup>+</sup> antiporter during cell volume regulation. *J. biol. Chem.* **267**, 23823–23828.
- HENIKOFF, S., HAUGHN, G. W., CALVO, J. M. AND WALLACE, J. C. (1988). A large family of bacterial activator proteins. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6602–6606.
- IVEY, D. M., GUFFANTI, A. A., BOSSEWITCH, J. S., PADAN, E. AND KRULWICH, T. A. (1991). Molecular cloning and sequencing of a gene from alkaliphilic *Bacillus firmus* OF4 that functionally complements an *Escherichia coli* strain carrying a deletion in the *nhaA* Na<sup>+</sup>/H<sup>+</sup> antiporter gene. *J. biol. Chem.* **266**, 23483–23489.
- IVEY, D. M., GUFFANTI, A. A., ZEMSKY, J., PINNER, E., KARPEL, R., PADAN, E., SCHULDINER, S. AND KRULWICH, T. A. (1993). Cloning and characterization of a putative Ca<sup>++</sup>/H<sup>+</sup> antiporter gene from *Escherichia coli* upon functional complementation of Na<sup>+</sup>/H<sup>+</sup> antiporter deficient strains by the overexpressed gene. *J. biol. Chem.* **268**, 11296–11303.
- JIA, Z. P., MCCULLOUGH, N., MARTEL, R., HEMMINGSEN, S. AND YOUNG, P. G. (1992). Gene amplification at a locus encoding a putative Na<sup>+</sup>/H<sup>+</sup> antiporter confers sodium and lithium tolerance in fission yeast. *EMBO J.* **11**, 1631–1640.
- KABACK, H. R. (1988). Site-directed mutagenesis and ion-gradient driven active transport: on the path of the proton. *A. Rev. Physiol.* **50**, 243–256.
- KARPEL, R., ALON, T., GLASER, G., SCHULDINER, S. AND PADAN, E. (1991). Expression of a sodium proton antiporter (*NhaA*) in *Escherichia coli* is induced by Na<sup>+</sup> and Li<sup>+</sup> ions. *J. biol. Chem.* **266**, 21753–21759.
- KARPEL, R., OLAMI, Y., TAGLICHT, D., SCHULDINER, S. AND PADAN, E. (1988). Sequencing of the gene *ant* which effects the Na<sup>+</sup>/H<sup>+</sup> antiporter activity in *E. coli*. *J. biol. Chem.* **263**, 10408–10414.
- KRULWICH, T. A., GUFFANTI, A. A. AND SETO-YOUNG, D. (1990). pH homeostasis and bioenergetic work in alkalophiles. *FEMS Microbiol. Rev.* **75**, 271–278.
- KRULWICH, T. A., HICKS, D. B., SETO-YOUNG, D. AND GUFFANTI, A. A. (1988). The bioenergetics of alkaliphilic bacilli. *Crit. Rev. Microbiol.* **16**, 15–36.
- MALONEY, P. C. (1992). The molecular and cell biology of anion transport by bacteria. *BioEssays* **14**, 757–762.
- NICOLL, D. A., LONGONI, S. AND PHILIPSON, K. D. (1990). Molecular cloning and functional expression of the cardiac sarcolemmal Na<sup>+</sup>/Ca<sup>++</sup> exchanger. *Science* **250**, 562–565.
- NIHIA, S., YAMASAKI, K., WILSON, T. H. AND TSUCHIYA, T. (1982). Altered cation coupling to melibiose transport in mutants of *Escherichia coli*. *J. biol. Chem.* **257**, 8902–8906.
- OLSNES, S., TONNESSEN, T. I., LUDT, J. AND SANDVIG, K. (1987). Effect of intracellular pH on the rate of chloride uptake and efflux in different mammalian cell lines. *Biochemistry, N.Y.* **26**, 2778–2785.
- PADAN, E., MAISLER, N., TAGLICHT, D., KARPEL, R. AND SCHULDINER, S. (1989). Deletion of *ant* in *E. coli* reveals its function in adaptation to high salinity and an alternative Na<sup>+</sup>/H<sup>+</sup> antiporter system(s). *J. biol. Chem.* **264**, 20297–20302.
- PADAN, E., SARKAR, H. K., VIITANEN, P. V., POONIAN, M. S. AND KABACK, H. R. (1985). Site-specific mutagenesis of histidine residues in the *lac* permease of *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6765–6768.
- PADAN, E. AND SCHULDINER, S. (1986). Intracellular pH regulation in bacterial cells. *Meth. Enzymol.* **125**, 337–352.
- PADAN, E. AND SCHULDINER, S. (1987). Intracellular pH and membrane potentials as regulators in the prokaryotic cell. *J. Membr. Biol.* **95**, 189–198.
- PADAN, E. AND SCHULDINER, S. (1992). Na<sup>+</sup>-transport systems in prokaryotes. In *Alkali Cation Transport Systems in Prokaryotes* (ed. E. Bakker), pp. 3–24. Boca Raton, FL: CRC Press.
- PADAN, E. AND SCHULDINER, S. (1993). Na<sup>+</sup>/H<sup>+</sup> antiporters, molecular devices that couple the Na<sup>+</sup> and H<sup>+</sup> circulation in cells. *J. Bioenerg. Biomembr.* **25**, 647–669.
- PADAN, E. AND SCHULDINER, S. (1994). Molecular physiology of Na<sup>+</sup>/H<sup>+</sup> antiporters, key transporters in circulation of Na<sup>+</sup> and H<sup>+</sup> in cells. *Biochim. biophys. Acta* **1185**, 129–151.
- PADAN, E., ZILBERSTEIN, D. AND ROTTENBERG, H. (1976). The proton electro-chemical gradient in *E. coli*. *Eur. J. Biochem.* **63**, 533–541.
- PADAN, E., ZILBERSTEIN, D. AND SCHULDINER, S. (1981). pH homeostasis in bacteria. *Biochim. biophys. Acta* **650**, 151–166.

- PINNER, E., KOTLER, Y., PADAN, E. AND SCHULDINER, S. (1993). Physiological role of NhaB, a specific Na<sup>+</sup>/H<sup>+</sup> antiporter in *Escherichia coli*. *J. biol. Chem.* **268**, 1729–1734.
- PINNER, E., PADAN, E. AND SCHULDINER, S. (1992). Cloning, sequencing and expression of *nhaB* gene, encoding a Na<sup>+</sup>/H<sup>+</sup> antiporter in *Escherichia coli*. *J. biol. Chem.* **267**, 11064–11068.
- POOLMAN, B. AND KONINGS, W. N. (1993). Secondary solute transport in bacteria. *Biochim. biophys. Acta* **1183**, 5–39.
- RAHAV-MANOR, O., CARMEL, O., KARPEL, R., TAGLICHT, D., GLASER, G., SCHULDINER, S. AND PADAN, E. (1992). NhaR, a protein homologous to a family of bacterial regulatory proteins (LysR) regulates *nhaA*, the sodium proton antiporter gene in *Escherichia coli*. *J. biol. Chem.* **267**, 10433–10438.
- RALEY-SUSMAN, K. M., CRAGOE, E. J., JR, SAPOLSKY, R. M. AND KOPITO, R. R. (1991). Regulation of intracellular pH in cultured hippocampal neurones by an amiloride-insensitive Na<sup>+</sup>/H<sup>+</sup> exchanger. *J. biol. Chem.* **266**, 2739–2745.
- ROSEN, B. P. (1986). Recent advances in bacterial ion transport. *A. Rev. Microbiol.* **40**, 263–286.
- SARDET, C., COUNILLON, L., FRANCHI, A. AND POUYSSEGUR, J. (1990). Growth factors induce phosphorylation of the Na<sup>+</sup>/H<sup>+</sup> antiporter, a glycoprotein of 110 kD. *Science* **247**, 723–726.
- SARDET, C., FAFOURNOUX, P. AND POUYSSEGUR, J. (1991a). A-thrombin, EGF and okadaic acid activate the Na<sup>+</sup>/H<sup>+</sup> exchanger, NHE-1 by phosphorylating a set of common sites. *J. biol. Chem.* **29**, 19166–19171.
- SARDET, C., WAKABAYASHI, S., FAFOURNOUX, P. AND POUYSSEGUR, J. (1991b). Phosphorylation of the growth-factor activatable Na<sup>+</sup>/H<sup>+</sup> antiporter: a mechanism of regulation. In *Biological Signal Transduction* (ed. E. M. Ross and K. W. A. Wirtz), pp. 253–269. Springer-Verlag.
- SCHULDINER, S. AND PADAN, E. (1992). Na<sup>+</sup>/H<sup>+</sup> antiporters in *Escherichia coli*. In *Alkali Cation Transport Systems in Prokaryotes* (ed. E. P. Bakker), pp. 25–51. Boca Raton, FL: CRC Press.
- SCHULDINER, S. AND PADAN, E. (1993). Molecular analysis of the role of Na<sup>+</sup>/H<sup>+</sup> antiporters in bacterial cell physiology. *Int. Rev. Cytol.* **137C**, 229–266.
- SKULACHEV, V. P. (1984). Sodium bioenergetics. *Trends biol. Sci.* **9**, 483–485.
- SKULACHEV, V. P. (1988). The sodium world. In *Membrane Bioenergetics* (ed. V. P. Skulachev), pp. 293–326. Berlin: Springer-Verlag.
- SLONCZEWSKI, J. L., MACNAB, R. M., ALGER, J. R. AND CASTEL, A. (1982). Effects of pH and repellent tactic stimuli on protein methylation levels in *Escherichia coli*. *J. Bacteriol.* **152**, 384–399.
- SPEELMANS, G., POOLMAN, B., ABEE, T. AND KONINGS, W. N. (1993). Energy transduction in the thermophilic anaerobic bacterium *Clostridium fervidus* is exclusively coupled to sodium ions. *Proc. natn. Acad. Sci. U.S.A.* **90**, 7975–7979.
- STORZ, G., TARTAGLIA, L. A. AND AMES, B. N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248**, 189–194.
- SUKHAREV, S. I., BLOUNT, P., MARTINAC, B., BLATTNER, F. R. AND KUNG, C. (1994). A large conductance mechanosensitive channel in *E. coli* encoded by *mscL* alone. *Nature* **368**, 265–268.
- TAGLICHT, D., PADAN, E. AND SCHULDINER, S. (1991). Overproduction and purification of a functional Na<sup>+</sup>/H<sup>+</sup> antiporter coded by *nhaA* (*ant*) from *Escherichia coli*. *J. biol. Chem.* **266**, 11289–11294.
- TAGLICHT, D., PADAN, E. AND SCHULDINER, S. (1993). Proton–sodium stoichiometry of NhaA, an electrogenic antiporter from *Escherichia coli*. *J. biol. Chem.* **268**, 5382–5387.
- TOKUDA, H. (1992). The Na<sup>+</sup> cycle in *Vibrio alginolyticus*. In *Alkali Cation Transport Systems in Prokaryotes* (ed. E. P. Bakker), pp. 125–138. Boca Raton, FL: CRC Press.
- TOKUDA, H. AND UNEMOTO, T. (1985). The Na<sup>+</sup>-motive respiratory chain of marine bacteria. *Microbiol. Sci.* **2**, 65–71.
- TSE, M., LEVINE, S., YUN, C., BRANT, S., POUYSSEGUR, J. AND DONOWITZ, M. (1993). The mammalian Na<sup>+</sup>/H<sup>+</sup> exchanger gene family – initial structure function studies. *Am. Soc. Nephrol.* **4**, 969–975.
- WASER, M., HESS-BIENZ, D., DAVIES, K. AND SOLIOZ, M. (1992). Cloning and disruption of a putative Na/H-antiporter gene of *Enterococcus hirae*. *J. biol. Chem.* **267**, 5396–5400.