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THE ROLE OF MONOVALENT CATION/PROTON ANTIPORTERS IN Na^+-RESISTANCE AND pH HOMEOSTASIS IN *BACILLUS*: AN ALKALIPHILE *VERSUS* A NEUTRALOPHILE

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Summary

Both neutralophilic *Bacillus subtilis* and alkaliphilic *Bacillus firmus* OF4 depend upon electrogenic Na^+/H^+ antiporters, which are energized by the gradients established by respiration-coupled proton extrusion, to achieve Na^+-resistance and pH homeostasis when the external pH is very alkaline. The interplay of proton and sodium cycles is discussed. In *B. subtilis*, pH homeostasis, up to pH 9, can be achieved using K^+ when Na^+ is unavailable or when the gene encoding the Na^+/H^+ antiporter that is involved in Na^+-dependent pH homeostasis is disrupted. That gene is a member of the tetracycline efflux family of genes. A second gene, encoding a Na^+/H^+ antiporter that functions in Na^+-resistance, has been identified, and candidates for the K^+/H^+ antiporter genes are under investigation. Aggregate Na^+/H^+ antiport activity in *B. subtilis* is as much as 10 times lower than in the alkaliphile, and the neutralophile cannot regulate its internal pH upon a shift to pH 10.5. Upon such a shift, there is a pronounced reduction in the generation of a primary electrochemical proton gradient. The alkaliphile, by contrast, maintains substantial driving forces and regulates its internal pH in an exclusively Na^+-coupled manner upon shifts to either pH 8.7 or 10.5. One gene locus has been identified and a second locus has been inferred as encoding relevant antiporter activities.

Prokaryotic patterns of Na^+ translocation relevant to Na^+-resistance or pH homeostasis

Monovalent cation/proton antiporters, and Na^+/H^+ antiporters in particular, have been known or proposed to play a large variety of important physiological roles, including resistance to elevated levels of Na^+ in the medium, pH homeostasis, osmoregulation and signalling (Krulwich, 1983; Grinstein *et al.* 1992; Wakabayashi *et al.* 1992; Schuldiner and Padan, 1993). General patterns (Fig. 1–3) have emerged with respect to the interplay between the sodium cycles and proton cycles that usually co-exist in bacterial cells.

Neutralophiles

For neutralophilic prokaryotes (Fig. 1), such as *Escherichia coli* and *Bacillus subtilis*, there is a primary proton cycle whereby outward proton pumping during respiration establishes an electrochemical gradient of protons, Δp, positive and acid outside relative

Key words: alkaliphile, *Bacillus subtilis*, *Bacillus firmus* OF4, Na^+/H^+ antiporter, K^+/H^+ antiporter, tetracycline efflux pump.

Primary H^+ cycle, H^+- and Na^+-coupled secondary porters

Neutralophilic, non-marine prokaryotes, some of which may exhibit primary Na^+ cycles under specific conditions of environmental challenge

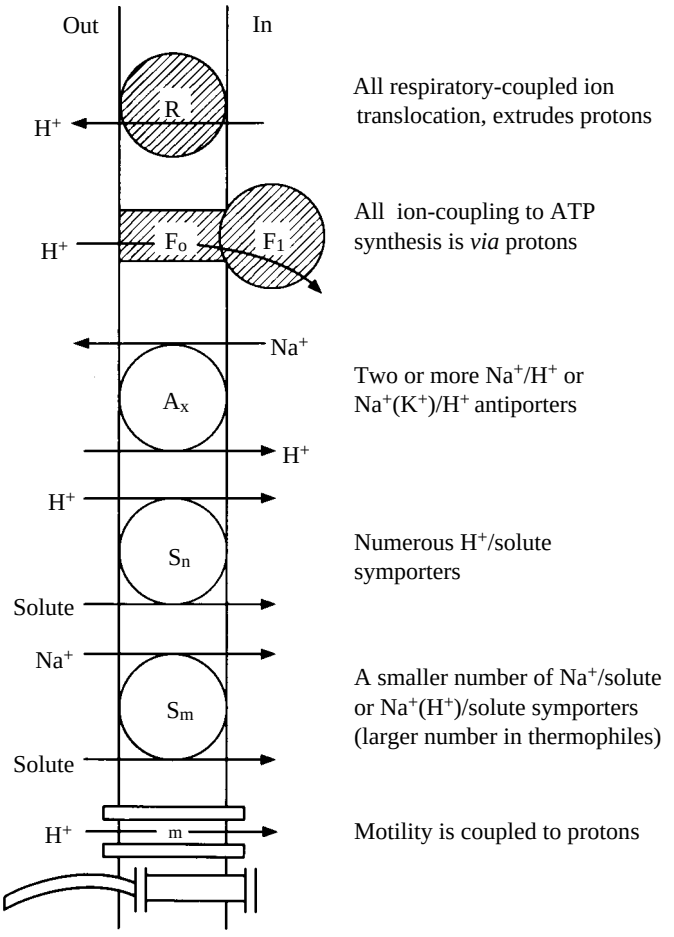


Fig. 1. Patterns of interactive proton and sodium cycles in neutralophilic non-marine prokaryotes.

to the cytoplasm. The Δp is the direct energy source for proton-coupled solute symporters, for proton-coupled ATP synthesis *via* the F_1F_o-ATP synthase and for motility. Evidence has been presented for a primary Na^+ cycle in *E. coli*, consisting of a Na^+-translocating respiratory chain complex, perhaps cytochrome *d* (Avetisyan *et al.* 1992), that might be coupled to a Na^+-translocating ATP synthase. Were such a cycle to be induced when specific conditions preclude the development of a large Δp, it might indeed give an energetic boost (Avetisyan *et al.* 1991). However, the evidence to date does not include a biochemical demonstration of the putative Na^+-coupled porters or a definition of the gene products that either encode the new functions or encode modulators that alter the cation specificity of known porters; nor has it yet been shown that Na^+/H^+-antiporter-deficient strains exhibit the phenomenon.

It is clearly established that the Δp produced *via* respiration energizes secondary antiporters, including Na^+/H^+ and K^+/H^+ antiporters that are often electrogenic (Padan and Schuldiner, 1993*a*,*b*). Electrogenic antiporters, such as NhaA in *E. coli* (Padan and Schuldiner, 1993*b*), could take energetic advantage of the transmembrane electrical gradient ($\Delta\Psi$) component of the Δp, and are thus promising candidates for major roles in pH homeostasis in the alkaline range of pH. Operation of Na^+/H^+ antiporters would also result in the generation of an inwardly directed Na^+ gradient, which could energize any Na^+-coupled solute uptake systems. Macnab and Castle (1987) presented a model of pH-dependent actions of multiple antiporters that, together, could account for observed patterns of Δp generation. A further analysis has been done on *E. coli* (Padan and Schuldiner, 1993*a*), based on rapidly emerging experimental data from specific deletion mutants (Padan *et al.* 1989; Thelen *et al.* 1991; Ohyama *et al.* 1992; Pinner *et al.* 1993).

In both *E. coli* and *B. subtilis*, growth becomes distinctly suboptimal at pH values of about 9 and above, on well-buffered media containing non-fermentative carbon sources. Moreover, while each of these neutralophiles is capable of growth in the presence of 700 mmol l^{-1} NaCl or more at pH 7, there is a progressively greater sensitivity to inhibition by NaCl as the growth pH is raised; often there is concomitant Li^+-sensitivity, but not K^+-sensitivity. The Na^+-sensitivity is increased in mutants that are deficient in Na^+/H^+ antiporters (Padan *et al.* 1989; Pinner *et al.* 1993, and see below). Thus, a role for Na^+/H^+ antiporters in Na^+-resistance is clear in both *E. coli* and *B. subtilis*, with sensitivity being most pronounced at elevated values of the growth pH. Less clear for *E. coli* is a role for Na^+/H^+ antiporters in growth at high pH in the absence of appreciable Na^+, since even a mutant with double deletions in genes encoding these porters can still grow at pH 8.5 in the absence of added Na^+ (Pinner *et al.* 1993). However, it is possible that if other cations, whose antiport with protons might offer a substitute, were restricted, or if the genes encoding those other porters were deleted, it would be shown that either Na^+/H^+ antiport or some substitute antiport is required for pH homeostasis (Padan and Schuldiner, 1993*b*). As described below, recent studies from our laboratory indicate that this is the situation in *B. subtilis.*

Alkaliphiles

Extremely alkaliphilic *Bacillus* species, such as *Bacillus firmus* OF4, that have also been studied in our laboratory, exhibit a variation of the above pattern (Fig. 2). Respiration and ATP synthesis are entirely H^+-coupled, albeit perhaps requiring a special pathway for the proton moving between the respiratory chain pump and the synthase (Krulwich and Guffanti, 1989, 1992). Secondary, electrogenic Na^+/H^+ antiporter activity allows the alkaliphiles to generate a large pH gradient, acid inside, relative to the highly alkaline exterior, and has been the most clear-cut example of a role of Na^+/H^+ antiporters in pH homeostasis (Krulwich and Guffanti, 1989, 1992). The ΔpH in alkaliphiles (2 pH units) is larger than that found in neutralophiles; moreover, the ΔpH is in the reverse direction to that found (Sturr *et al.* 1994) over most of the pH range for growth of neutralophiles (Krulwich and Ivey, 1990). A non-alkaliphilic mutant phenotype that results in an inability to regulate cytoplasmic pH upon a shift to pH 10.5 has always been associated with a reduction in electrogenic Na^+ extrusion in exchange for H^+ (Krulwich

Primary H^+ cycle, extraordinarily active, and exclusively Na^+-coupled secondary porters

Extreme alkaliphiles, extreme halophiles also have exclusively H^+-coupled primary cycle and extensive Na^+-coupled secondary cycle

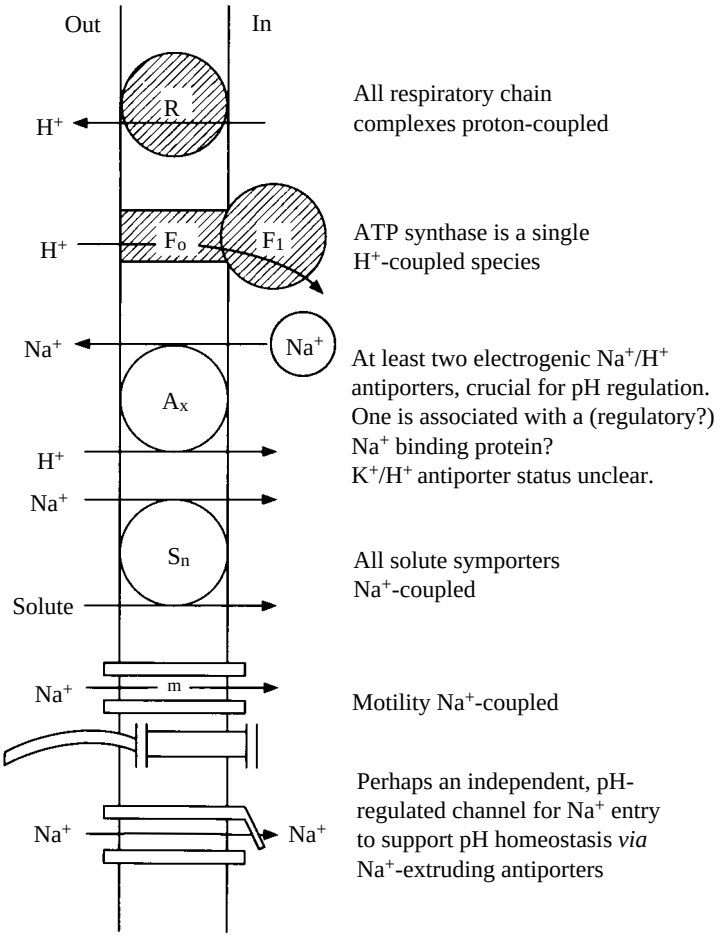


Fig. 2. Patterns of interactive proton and sodium cycles in extreme alkaliphiles.

and Guffanti, 1989). Also, in contrast to neutralophiles, all solute symporters of alkaliphiles are Na^+-coupled (Krulwich and Guffanti, 1989), as is motility (Hirota *et al.* 1981). The solute symporters, and perhaps the ion channel that is putatively associated with motility, provide a re-entry route for Na^+, so that Na^+ extrusion in exchange for H^+ can be sustained. Booth (1985) suggested that additional pH-dependent channels for Na^+ are also present and account for antiporter activity and pH homeostasis occurring in the absence of obvious solutes, whose uptake would facilitate Na^+ re-entry. Mechanosensitive channels have been characterized in *E. coli* membranes (Sukharev *et al.* 1994) and alkaliphiles might have a pH-responsive equivalent. In alkaliphilic *B. firmus*, no specific Na^+ channels have yet been identified, but pH homeostasis is

strikingly enhanced by the presence of a non-metabolizable solute whose entry is coupled to Na^+ entry (Krulwich *et al.* 1985). Extreme halophiles, interestingly, share most of the general pattern shown for alkaliphiles in Fig. 2, although the major biological challenge is quite different.

Marine and anaerobic bacteria

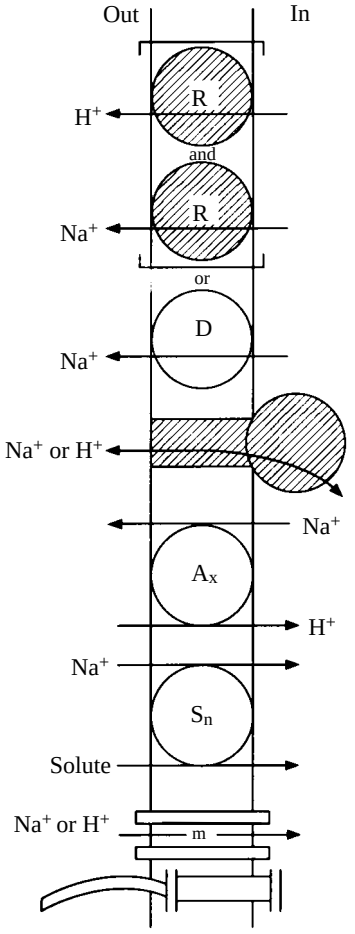
A third general pattern includes marine organisms and a number of anaerobes with specialized Na^+-translocating elements found together with H^+-coupled elements (Fig. 3). Examples include two anaerobic bacteria that possess Na^+-translocating F_1F_o-ATPases: marine *Priopionigenium modestum* (Dimroth, 1990) and homoacetogenic *Acetobacterium woodii* (Heise *et al.* 1992). *P. modestum* also possesses membrane-associated, Na^+-translocating decarboxylases (Dimroth, 1990). Probably both *P. modestum* and *A. woodii* will also be found to have at least one Na^+/H^+ antiporter. In *Vibrio* species (Unemoto *et al.* 1990) and other marine bacteria, such as alkali-tolerant *Bacillus* FTU (Semeykina and Skulachev, 1992), there are respiratory complexes that translocate Na^+ side-by-side with ones that translocate protons; these organisms may also have Na^+-coupled ATPases. However, purified and reconstituted F_1F_o-ATPase from *V. alginolyticus* is exclusively H^+-coupled (Krumholz *et al.* 1990; Dimitriev *et al.* 1992); the nature of the apparent Na^+-coupled ATPase is unclear. The Na^+-translocating primary pump(s) enhance growth under conditions in which the Δp is reduced (Unemoto *et al.* 1990; Semeykina and Skulachev, 1992), but the degree to which the boost involves ATP synthesis *versus* support of Na^+ extrusion, which would generate an electrochemical Na^+ gradient and enhance solute uptake, is uncertain. Roles for both Na^+/H^+ and K^+/H^+ antiport have been proposed in the pH homeostasis of *V. alginolyticus* (Nakamura *et al.* 1992), and the gene encoding an apparent Na^+/H^+ antiporter has recently been cloned (Nakamura *et al.* 1994). In non-respiring *Enterococcus hirae*, both a Na^+/H^+ antiporter (Waser *et al.* 1992) and a Na^+-translocating, V-type ATPase (Takase *et al.* 1993) are involved in Na^+-resistance. The primary pump may also be part of an adaptation to a low Δp, either by generating a Na^+ gradient for coupling to solute transport, or by providing a substitute Na^+ efflux mechanism for Δp-dependent antiport when Δp generation by the primary H^+-coupled ATPase is insufficient. Another group of organisms that shares features with this third general category are the methanogens (Blaut *et al.* 1992). Omitted from the summary in Figs 1–3 are *Clostridium fervidus*, which is entirely Na^+-coupled, lacking even Na^+/H^+ antiport (Speelmans *et al.* 1993), and acidophilic bacteria. Acidophiles achieve a characteristic Δp pattern of a large ΔpH, acid outside, and the unusual $\Delta\Psi$, positive inside, at low external pH, probably without the necessity for novel fluxes; their bulk Δp is also apparently sufficient to energize ATP synthesis by conventional mechanisms *via* a proton-translocating ATP synthase (Ingledew, 1990; Krulwich and Ivey, 1990).

What might be the basis for the far greater capacity of alkaliphiles for pH homeostasis than that of a neutralophile?

Recent studies of the bioenergetic variables of *B. firmus* OF4 growing in continuous cultures at various rigorously controlled external pH values underscore the alkaliphile's

Primary Na^+ cycle, alone or as alternative to primary H^+ cycle, secondary Na^+-coupled porters

Marine and other specialized, often facultative or obligate, anaerobes, some alkaline-tolerant



Under at least some growth conditions special, Na^+-translocating respiratory chain complexes function

Anaerobic catabolism utilizes primary Na^+-translocating decarboxylases

A Na^+-translocating ATPase: in some instances an F-type synthase that also couples to H^+; otherwise, the Na^+-ATPase is not entirely Na^+-extruding and co-exists with an H^+-coupled F-ATPase

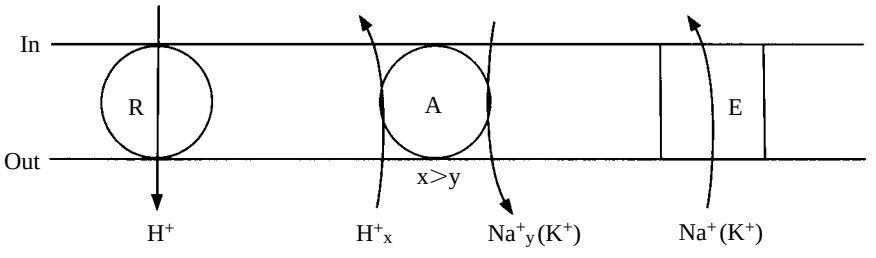
At least one Na^+/H^+ antiporter

Most, if not all, symporters coupled to Na^+

Motility in some of these organisms is coupled to Na^+ exclusively or to either H^+ or Na^+, depending upon the conditions or flagellar type

Fig. 3. Patterns of interactive proton and sodium cycles in marine and other specialized anaerobes.

remarkable capacity for pH regulation and show that it is, in fact, this capacity that is ultimately limiting to growth at the upper edge of the pH range, at around pH 11.2–11.4 (Sturr *et al.* 1994). Cells generate a progressively larger ΔpH, acid inside, as the external pH rises from 7.5 to 9.5, accompanied, and presumably energized, by a $\Delta\Psi$ that increases from -140 mV to just over -180 mV (inside negative). At higher pH values of the growth medium, both the $\Delta\Psi$ and the ΔpH remain almost constant, with the ΔpH between 2 and 2.3 units, acid inside, finally decreasing only to 1.8 units at pH 11.4. Accordingly, the cytoplasmic pH climbs progressively to 8.8 at an external pH of 11.2 and a bit more sharply, to 9.5, at an external pH of 11.4. Perhaps the rather constant maximal ΔpH of about 2 units reflects a thermodynamic limit on the magnitude of the



Are the cations used for the near-neutral alkaline range the same as those used in the extreme alkaline range?

What limits the capacity for pH homeostasis as pHe is raised?

Is it the same for an alkaliphile as for a neutralophile?

Fig. 4. Elements catalyzing ion fluxes that together constitute a bacterial pH homeostatic mechanism. R is the H^+-extruding respiratory chain that establishes the primary proton gradient (Δp) in both alkaliphilic and neutralophilic bacteria. A is the electrogenic monovalent cation/proton antiporters. E marks the entry pathways for the cations extruded during antiport. No precise mechanisms or stoichiometries are specified, or are as yet known, for these elements. pHe, extracellular pH.

ΔpH that can be generated, defined by the stoichiometry of the aggregate antiport above pH 9.5 and the magnitude of $\Delta\Psi$.

The elements of the H^+ and Na^+ cycles that are involved in pH homeostasis in the alkaline range of pH are shown schematically in Fig. 4. For a given organism under different conditions, or for different organisms, a different set of elements might be limiting. For example, in batch cultures that are in lag phase, the concentration of antiporters in the membrane might limit pH homeostasis until full induction has occurred. Variants of *B. firmus* that can grow better than the wild-type strain at very high initial pH values had elevated antiporter activity (Krulwich *et al.* 1986). Such variants appear to express their antiport complement constitutively rather than to have higher levels than the wild type when both are fully induced (M. G. Sturr, unpublished data). In pH-shift experiments with a different alkaliphile, McLaggan *et al.* (1984) established conditions in which pH homeostasis by fully induced, energized cells was dependent upon the sodium concentration. In such experiments, it is not simple to determine whether antiport itself or Na^+ re-entry is limiting (A and E respectively in Fig. 4), even if initial cation efflux is monitored from pre-loaded cells or vesicles. In pH-shift experiments with *B. firmus* RAB, pH homeostasis at high Na^+ concentrations appeared to be limited by Na^+ entry only after the first few minutes post-shift, whereas at low Na^+ concentrations, Na^+ entry was immediately limiting (Krulwich *et al.* 1985).

Under some conditions, the magnitude of the primary driving force (R in Fig. 4) limits the rate or extent of cytoplasmic pH regulation in cells in which all the relevant antiporters are fully induced and activated. In cells pre-loaded with sodium, the initial rate

of diffusion-potential-induced, antiporter-mediated $^{22}Na^+$ efflux from alkaliphile cells grown at high pH is proportional to the magnitude of the potential imposed (Garcia *et al.* 1983). Similarly, if alkaliphile respiration is submaximal, so is Na^+-dependent pH homeostasis. Hoffmann and Dimroth (1991*a*) reported that a strain of *B. alcalophilus* from the DSM collection could not grow well at pH values above 9.5, although excellent growth of the ATCC strain of *B. alcalophilus* at pH 10.5 had been found (Guffanti *et al.* 1978). It turned out that the DSM strain required fresh medium to produce normal cytochrome levels, probably because of an auxotrophy related to iron sequestration. Growth of the DSM and ATCC strains at pH 10.5 was the same in continuous culture and at early points in batch culture (Guffanti and Hicks, 1991; Hoffmann and Dimroth, 1991*b*). The lower cytochrome contents later in the growth curve correlated, at least when measured using standard approaches (Krulwich and Guffanti, 1992), with a lower $\Delta\Psi$ and with the diminished growth capacity at high pH (Guffanti and Hicks, 1991; Hoffmann and Dimroth, 1991*a*). These considerations illustrate the importance of evaluating new proposals for primary Na^+ cycles from the point of view that the effects observed might result from enhancement of the primary proton potential that then acts to increase secondary antiport.

An organism that is apparently capable of remarkable pH homeostasis might differ from a conventional one in any one or more of the three distinct elements shown in Fig. 4. *B. subtilis* and alkaliphilic *B. firmus* OF4 have vastly different upper pH limits for growth on non-fermentable carbon sources. Growth in the upper pH range for *B. subtilis* is dependent upon substantial concentrations (25–100 mmol l^{-1}) of either Na^+ or K^+, whereas for *B. firmus* OF4, Na^+ is strictly required. These differences correlated with behaviour in recent experiments (A. A. Guffanti, unpublished data) in which the two species were subjected to sudden upward shifts in the pH of the medium. Both species could maintain a cytoplasmic pH between 7.2 and 7.4 upon a shift of the medium pH from 7.3 to 8.7; whereas *B. subtilis* required either K^+ or Na^+, the alkaliphile required specifically Na^+. *B. subtilis* was incapable of acidifying its cytoplasm relative to the exterior upon a shift from pH 8.5 to 10.5; respiration-dependent generation of the primary Δp is at least partially responsible for the failure. However, the data are insufficient to distinguish between diminished respiratory activity or membrane leakiness as the cause of the lower $\Delta\Psi$, or to assess the possible concomitant loss of antiporter activity. By contrast, the alkaliphile exhibits pH homeostasis and $\Delta\Psi$ generation that is highly effective at pH 10.5. It is probably relevant that the aggregate membrane Na^+/H^+ antiport activity of *B. subtilis* was at least 10-fold lower than that of *B. firmus* OF4 when assayed by malate-dependent $^{22}Na^+$ efflux from pre-loaded cells at 21 °C at pH 8.8–9.

What specific molecular information is available about the antiporter complements of *Bacillus subtilis* and *Bacillus firmus* OF4?

Bacillus subtilis

In order to identify genes encoding antiporters that were important for pH homeostasis, for Na^+-resistance or for both, transposition libraries of *B. subtilis* that had been generated by transposition with Tn*917* (Quirk *et al.* 1993) were screened for a Na^+- and/or alkali-

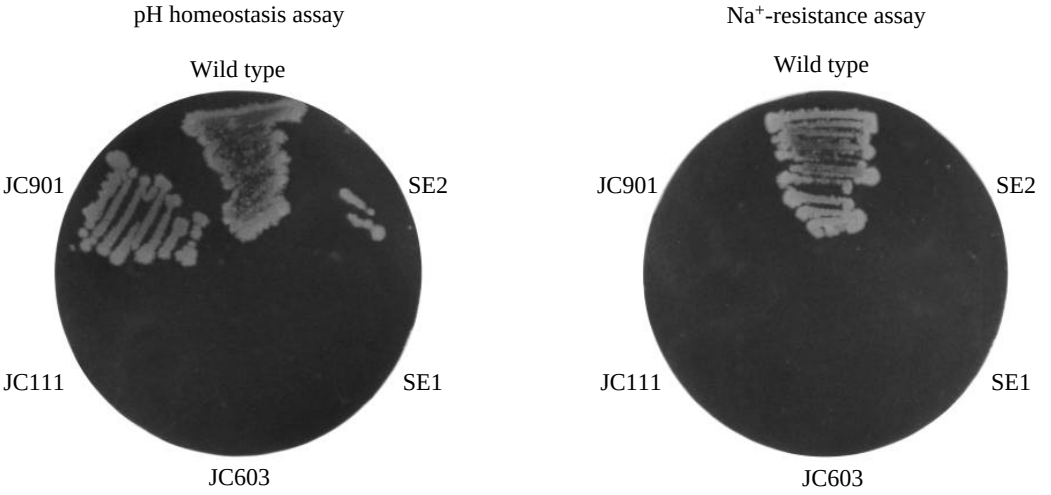


Fig. 5. The growth of wild-type *Bacillus subtilis* and five transposition mutants selected on the basis of Na^+-sensitivity at pH 8.5. (A) An illustration of the 'pH homeostasis' assay, with growth on Tris-malate-containing medium plus 50 mmol l^{-1} NaCl at pH 8.5. (B) An illustration of the 'Na^+-sensitivity' assay, which was growth on potassium-malate-containing medium plus 100 mmol l^{-1} NaCl at pH 8.5. The cells were grown overnight.

sensitive phenotype. Five mutant strains, each with a single transposon in the chromosome, have been the focus of subsequent studies. As shown in Fig. 5, none of these strains is capable of growth under conditions that have been developed as an index of Na^+-sensitivity, i.e. growth on potassium-malate-containing medium at pH 8.5 in the presence of 100 mmol l^{-1} added NaCl. One of the five strains, JC901, was capable of growing almost as well as the wild type under conditions developed as an index of the capacity for Na^+-dependent pH homeostasis at pH 8.5. This medium, at pH 8.5, contained Tris-malate as carbon source, a low concentration (1 mmol l^{-1}) of potassium phosphate, to provide enough potassium to support growth, and 50 mmol l^{-1} NaCl. Other experiments show that, if the NaCl is omitted, the wild type barely grows and is apparently unable to acidify its cytoplasm relative to the outside. When a higher concentration of potassium is included, the Na^+-dependence of growth at pH 8.5 disappears (J. Cheng, unpublished data). More detailed phenotypic studies suggest that the five strains shown are distinct from one another, but the insertion site of the transposon has only been characterized so far in JC901, JC111 and JC603.

In JC901, the transposon disrupts an apparent structural gene for a Na^+/H^+ antiporter with significant homology to eukaryotic Na^+/H^+ antiporters; disruption of the gene in JC901 reduces the total energy-coupled Na^+ efflux from whole cells (J. Cheng, unpublished data). The gene is expressed constitutively at low levels, so that this gene is tentatively named *nha*B, by analogy with the *E. coli* gene (Pinner *et al.* 1993). However, while disruption of *B. subtilis nha*B is not associated with defective pH homeostasis, it is unlike *E. coli nha*B in being associated with pronounced Na^+-sensitivity at elevated pH (Fig. 5, J. Cheng, unpublished data). A second antiporter gene is disrupted in JC111, producing a phenotype in which both Na^+-sensitivity and a loss of Na^+-dependent pH

homeostasis are evident. Since K^+-dependent pH homeostasis is little affected in JC111, and unaffected in JC901, as yet uncharacterized genes must account for this function.

Interestingly, the gene locus disrupted in JC111 is one previously identified as the *tet*B locus of *B. subtilis* (Williams and Smith, 1979). Although wild-type *B. subtilis*, containing a single chromosomal *tet*B gene, is tetracycline-sensitive when tested with usual challenge concentrations, amplification of the gene by specific growth protocols (Wilson and Morgan, 1985; Ives and Bott, 1990) or on plasmids (Sakaguchi and Shishido, 1988; Ives and Bott, 1989) results in tetracycline-resistance. The deduced sequence of *tet*B clearly resembles those of the tetracycline efflux family of proteins (Sakaguchi *et al.* 1988; Schwarz *et al.* 1992). The studies of JC111 now suggest that the physiological role of TetB is as an Nha-like Na^+/H^+ antiporter that functions both in Na^+-dependent pH homeostasis and in Na^+-resistance in *B. subtilis* (J. Cheng, unpublished data). These findings are of interest in connection with the origins of this class of antibiotic-resistance determinants and with respect to the biochemistry of the transporters with diverse substrates. In addition, if *tet*B is really a *B. subtilis* equivalent of *E. coli nha*A, the regulation of this locus needs to be sorted out, since it is currently thought likely to be controlled by antibiotic-dependent translational effects (Schwarz *et al.* 1992) by analogy with several other resistance loci (Dubnau, 1984; Lovett, 1990). However, induction at the transcriptional level has been reported for some plasmid *tet* genes (e.g. Mojumdar and Khan, 1988), and it will be of interest to determine whether Na^+ is an inducer. The instances of amplification of the *tet*B locus on the *B. subtilis* chromosome are also notable, a phenomenon that may depend upon its location near the origin of replication (Ives and Bott, 1989, 1990; Amano *et al.* 1991). Gene amplification might normally be part of the repertoire of adaptation to an alkaline pH and/or Na^+ challenge.

JC603 is another Na^+-sensitive transposition mutant, whose insertion is in a non-coding region on the other side of *tet*B from the origin. A speculative hypothesis with respect to JC603 is that the bulky transposon interferes with amplification of *tet*B. Might gene amplification also occur in *E. coli*, in which *nha*A is also very near the origin of replication (Schuldiner and Padan, 1993)?

In summary, the initial results of the transpositional analysis indicate that, in *B. subtilis*, Na^+-resistance depends upon at least two genes, those disrupted in JC111 and JC901, with additional candidates under study. The *tet*B gene on the *B. subtilis* chromosome functions in Na^+-dependent pH homeostasis as well, but other genes, perhaps those disrupted in SE1 and SE2, must account for K^+-dependent pH homeostasis. Either Na^+ or K^+ can support growth of *B. subtilis* at pH 8.5, but the wild-type strain will not grow on media deficient in both monovalent cations at pH 8.5, although growth on the same medium at pH 7 is excellent (J. Cheng, unpublished data).

Alkaliphilic Bacillus firmus *OF4*

Because of a current limitation in the genetic approaches available for the study of *B. firmus* OF4, the overall picture with respect to the likely number or specific characteristics of antiporters involved in pH homeostasis and/or Na^+-resistance is less clear in the alkaliphile than in *B. subtilis*. Two different loci are thus far implicated. Genes encoding Na^+/H^+ antiporters in *B. firmus* OF4 were sought by using plasmid libraries of

alkaliphile DNA to complement functionally a mutant of *E. coli*, strain NM81 (Padan *et al.* 1989), that was deleted in *nha*A, rendering it Na^+-sensitive at pH 7.5 and Li^+-sensitive in the presence of melibiose. Complementing regions of the alkaliphile chromosome fell into two categories. First, the screen yielded two different putative cation-binding regulatory genes that do not complement by enhancing antiport activity, since they function in double *nha* deletions of *E. coli*, in the absence of a detectable increase in the low level of residual monovalent cation/proton antiport (A. A. Guffanti, unpublished data). Nor are they in alkaliphile operons encoding antiporters. One of these genes was the *cad*C gene, which encodes a small Cd^{+2}-binding protein (Ivey *et al.* 1992). CadC may interact with the actual membrane-associated pump protein that is encoded by *cad*A from the same operon (Silver and Walderhaug, 1992), but it is also likely to function as a cation-responsive regulator (Huckle *et al.* 1993). The second newly identified regulatory region that complements antiporter-deficient *E. coli* is part of an operon that encodes alkaliphile haemoglobin-like proteins as well as a topoisomerase; only the regulatory gene is required for the complementation (M. G. Sturr, unpublished data). This gene has significant sequence homology with a highly polar regulatory protein from *Streptococcus gordonii* (Sulavik *et al.* 1992). The basis for complementation by this group of gene products is hypothesized to be their capacity to bind enough Na^+, when the gene is expressed from multicopy plasmids, to protect sensitive cell targets from inhibition by free cytoplasmic Na^+. The overproduced Na^+-binding regulatory protein would be protective in much the same way that metallothionein is thought to protect against heavy metal toxicity in higher systems (Kagi, 1991). This type of complementation screen might be generally useful to identify new cation-binding regulatory elements independently of their specific *in vivo* function.

The other group of complementing genes were those encoding alkaliphile antiporters, the original object of the investigation. These included one apparent antiporter gene that has not been stably cloned yet, but has integrated into the *E. coli* chromosome, upon which it markedly increased the membrane antiporter activity with Na^+ or Li^+ (Ivey *et al.* 1991, 1993). A second alkaliphile antiporter gene, *nha*C, is predicted to encode a membrane protein with 10 membrane-spanning regions and with modest similarity to eukaryotic Na^+/H^+ antiporters (Ivey *et al.* 1991) and to other prokaryotic porters (Padan and Schuldiner, 1993*b*). As expressed in *E. coli*, the $Na^+(Li^+)/H^+$ antiporter activity conferred by expression of *nha*C is higher at pH 8.5 than at lower pH values and does not use K^+. There are two upstream candidates for promoter sequences, and downstream of *nha*C is an apparent Na^+-binding regulatory gene, *nha*S. As described for other Na^+-binding regulatory genes, clones expressing just *nha*S increase the Na^+-resistance of antiporter-deficient *E. coli* mutants without enhancing membrane antiport. Preliminary data suggest that NhaS may associate with the membrane under some conditions, where it could have a sodium-sensing role (J. Zemsky, unpublished data). It may share dual sensing and gene-activating roles with the NhaR regulatory protein for the NhaA antiporter of *E. coli* (Rahav-Manor *et al.* 1992), although *nha*S and *nha*R are structurally different types of proteins. Both the specific regulatory features and catalytic properties that underlie the alkaliphile's extremely high Na^+/H^+ antiport activity and remarkable capacity for pH homeostasis have yet to be clarified.

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