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MINI-REVIEW

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The Na^+/H^+ antiporter of alkaliphilic *Bacillus* sp.

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Abstract The Na^+/H^+ antiporter, which appears to predominantly contribute to the alkaliphily of *Bacillus halodurans* C-125, was studied in an alkali-sensitive mutant of this strain and a transformant with restored alkaliphily. The alkali-sensitive mutant, strain 38154, which has lost the ability to grow above pH 9.5, was found to lack electrogenic Na^+/H^+ antiport activity driven by $\Delta\Psi$ (membrane potential, interior negative), and it showed defective regulation of intracellular pH under alkaline conditions. On the other hand, a transformant carrying a 2.0-kb DNA fragment from the parental genome that complemented this defect was able to maintain an intracellular pH lower than that of the external milieu, and it was found to have recovered the Na^+/H^+ antiport activity driven by $\Delta\Psi$. Sequence analyses found that a 5.1-kb DNA region contained four open reading frames (ORF-1 to ORF-4). Direct sequencing of the corresponding region in mutant 38154 revealed a G-to-A substitution, which resulted in an amino acid substitution from Gly-393 to Arg in the putative ORF-1 product. It has been recently found that a region homologous to the DNA fragment responsible for the alkaliphily of strain C-125 exists in the genomes of *Bacillus subtilis*, *Sinorhizobium* (*Rhizobium*) *meliloti*, and *Staphylococcus aureus.* These homologues are present as a cluster of seven ORFs in each case. The *shaA* gene product of *B. subtilis* shows significant similarity to the ORF-1 product of strain C-125. Disruption of the *shaA* gene resulted in a decrease in Na^+/H^+ antiport activity, and growth of the *shaA*-disrupted strain was impaired when the external Na^+ concentration was increased. We conclude that the *shaA* gene encodes a Na^+/H^+ antiporter, which plays an important role in extrusion of cytotoxic Na^+.

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Introduction

Alkaliphiles require high pH and sodium ions for their growth. How alkaliphiles maintain a neutral cytoplasmic pH in alkaline environments, that is, pH homeostasis, is one of the most important topics in the study of alkaliphiles. Several different mechanisms have been suggested to play a role in such pH homeostasis (Krulwich et al. 1997). Aono et al. (1999) have recently shown that an acidic teichuronopeptide polymer in the cell wall serves as a barrier to flux of relevant ions and plays a role in pH homeostasis in the facultative alkaliphile *Bacillus* strain C-125. On the other hand, recent reports have suggested that Na^+/H^+ antiporters that operate electrogenically may play a predominant role in pH homeostasis (Guffanti and Krulwich 1980; Kitada et al. 1982; Krulwich et al. 1982; Krulwich 1986; Kitada et al. 1989). In this review, we describe the identification and characterization of a Na^+/H^+ antiporter, which is responsible for the alkaliphily of *Bacillus halodurans* C-125 (Takami and Horikoshi 1999). We have used strain C-125 as a model of alkaliphiles because (1) this strain grows over the pH range from pH 7.5 through 11 and requires sodium ions for growth; (2) it grows well in minimal medium, which is advantageous in selection of auxotrophic markers introduced into mutants of this strain; (3) a host–vector system has been developed using strain C-125; (4) many genes from this strain have been cloned, and it is evident that strain C-125 is closely related to *Bacillus subtilis*; thus, considerable *B. subtilis* data are available for use as control data in analysis of neutralophiles; and (5) analysis of the entire genome of strain C-125 is in progress.

As an approach taken in studying the mechanism(s) responsible for the alkaliphily of strain C-125, we isolated an alkali-sensitive mutant (38154) that had lost the ability to grow above pH 9.5 and which had concomitantly lost $\Delta\Psi$-driven electrogenic Na^+/H^+ antiport activity (Kudo et al.

1990). We then cloned a DNA fragment from the parental strain C-125 that restored the alkaliphily and the ΔΨ-dependent Na^+/H^+ antiport activity when introduced into mutant 38154 (Kitada et al. 1994; Hamamoto et al. 1994). This report was the first to show that a gene responsible for Na^+/H^+ antiport activity is important for alkaliphily in microorganisms. Homologous gene clusters have been found also in *B. subtilis*, *Sinorhizobium (Rhizobium) meliloti*, and *Staphylococcus aureus*, as described in a later section.

Isolation of alkali-sensitive mutants

Alkaliphiles can be divided into two groups: facultative alkaliphiles and obligate alkaliphiles. Facultative alkaliphiles show optimal growth at pH 10 or above, but can grow well in the neutral pH range. These facultative alkaliphiles are of particular interest because they offer an opportunity to use alkali-sensitive mutants experimentally (Krulwich and Guffanti 1989). Therefore, a facultative alkaliphile, *Bacillus halodurans* C-125, was used throughout our experiments. Alkali-sensitive mutants of strain C-125 were obtained by mutagenesis with nitrosoguanidine. Six alkali-sensitive mutants showing no growth at pH 10.5, although each grew well at pH 7.5, were obtained (Hashimoto et al. 1994). Only one of the mutants, 38154, was unable to sustain a low internal pH in an alkaline environment, whereas the other five mutants still retained this ability (Kudo et al. 1990; Hashimoto et al. 1994). We assumed that mutant 38154 had lost the function of pH homeostasis, important for alkaliphily, and further studied it.

It has been suggested that the Na^+/H^+ antiporter is involved in the regulation of internal pH in alkaliphiles and in *E. coli* when exposed to alkaline environments (Kitada et al. 1982; Zilberstein et al. 1982). There is general agreement that the Na^+/H^+ antiporter is driven by the ΔpH component or the ΔΨ component of the protonmotive force (PMF) (Bassilana et al. 1984a,b). However, alkaline pH is an adverse condition for generating ΔpH, and under such conditions only the ΔΨ component would contribute to the PMF. Thus, we examined the ΔΨ-dependent Na^+/H^+ antiport activity by measuring $^{22}Na^+$ efflux or H^+ influx in right-side-out membrane vesicles derived from the parent strain, and the mutant, under conditions where a ΔΨ (interior negative) was created by potassium diffusion in the presence of valinomycin (Kitada et al. 1994). The imposed ΔΨ led to intravesicular acidification in the parental strain C-125 but not in mutant 38154. Consistent with this result, in response to the imposed potential there was a rapid efflux of $^{22}Na^+$ from membrane vesicles of the parent strain but not from those of the mutant. These results indicate that this ΔΨ-dependent Na^+/H^+ antiporter catalyzes uphill H^+ influx coupled with Na^+ efflux when an artificial ΔΨ (interior negative) is imposed. Because the transmembrane potential imposed was equal in the case of the vesicles of both the parental and mutant strains, it can be concluded that the ΔΨ-dependent Na^+/H^+ antiporter in mutant 38154 was unable to function electrogenically at alkaline pH.

Isolation of a DNA fragment restoring alkaliphily in the alkali-sensitive mutant 38154

Using the alkali-sensitive mutant 38154 as a host for protoplast transformation, we tried to isolate DNA fragments that would restore the alkaliphily in this mutant. Chromosomal DNA from strain C-125 was digested with *Hin*dIII, ligated into the *Hin*dIII site of plasmid pHW1, and introduced into mutant 38154 via protoplast transformation. Transformants with restored alkaliphily were detected directly on plates of alkaline medium. The plasmid pALK2 isolated from such a transformant contained a 2.0-kb DNA insert in the *Hin*dIII site of pHW1, which appeared to be responsible for the recovery of alkaliphily. At pH 7.0–9.0, mutant 38154 could grow to the same extent as did the parental strain C-125, but beyond this range the extent of growth decreased with increasing pH, whereas strain C-125 and the transformant of strain 38154 harboring pALK2 could grow well over the pH range from 7 to 10.5. Furthermore, the transformant harboring pALK2 was able to maintain an internal pH lower than that of the external milieu and it was found to display ΔΨ-dependent Na^+/H^+ antiport activity driven by ΔΨ (Kudo et al. 1990). Therefore, it can be concluded that pALK2 contains genes encoding a ΔΨ-dependent Na^+/H^+ antiporter required for pH homeostasis in alkaline environments (Hamamoto et al. 1994).

Characterization of the gene responsible for Na^+/H^+ antiporter

Nucleotide sequence analysis showed that pALK2 contained parts of two ORFs (ORF-1 and ORF-3) and one complete ORF (ORF-2) (Fig. 1). To identify the region in pALK2 responsible for complementation of the defect in mutant 38154, three deleted derivatives of pALK2 were constructed and each was tested for the capacity to restore alkaliphilic growth in mutant 38154 (Hamamoto et al. 1994). As shown in Fig. 1, 38154 transformants harboring pALK2021 or pALK2101 grew well over the entire pH range from 6.5 to 11. On the other hand, the 38154 transformant harboring pALK2002 grew well only below pH 9. The ΔΨ-dependent Na^+/H^+ antiport activity expressed by each of these types of transformants was then examined by measuring H^+ influx into right-side-out membrane vesicles. Consistent with these growth phenotypes, imposing an artificial ΔΨ resulted in intravesicular acidification in the case of the 38154 (pALK2021) and 38154 (pALK2101) transformants, whereas Na^+-loaded vesicles derived from the 38154 (pALK2002) transformant exhibited no H^+ influx. These results indicate that the mutation site in the genome of mutant 38154 is in the DNA region

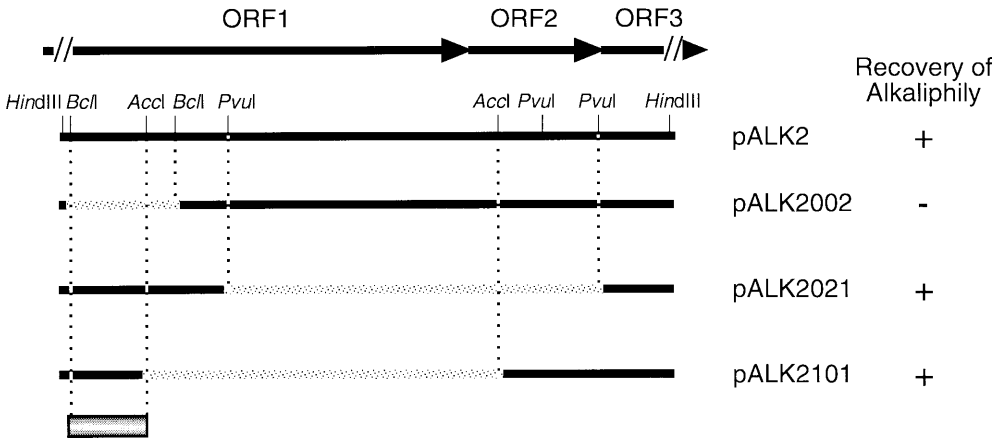

Fig. 1. Restriction maps of a DNA fragment from strain C-125 responsible for the recovery of alkaliphily in the 38154 mutant. A 2.0-kb *Hin*dIII fragment cloned in pALK2 is shown together with the open reading frames (*arrows*) located in the fragment. The subcloned parts of the *Hin*dIII fragment are indicated by the *thick line*. The regions shown by *dots* were deleted in subcloning of parts of the insert from pALK2. The effectiveness of each plasmid in restoring alkaliphily, as determined by testing transformants for the ability to grow in Horikoshi-II liquid medium (pH 10.0) at 37°C, is shown on the *right*. The DNA fragment that appears to be responsible for the recovery of alkaliphily is shown by a *shaded box*. (From data in Fig. 1 of Hamamoto et al. 1994)

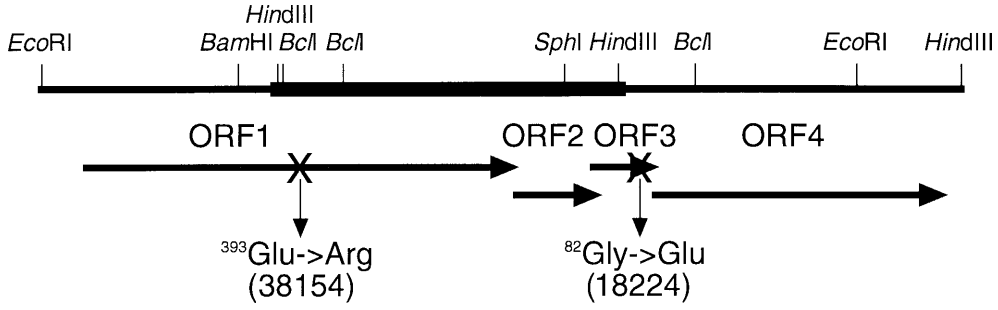

Fig. 2. Positions of amino acid substitutions responsible for the alkali sensitivity of mutants 38154 and 18224. A 5.1-kb *Eco*RI–*Hin*dII fragment from strain C-125 is shown together with ORF-1 to ORF-4 (*arrows*) located in the fragment. A *Hin*dIII fragment cloned in pALK2, which restores the alkaliphily of the mutant 38154, is indicated by a *thick line*. Amino acid substitutions from ^{393}Gly to Arg in the ORF-1 product and from ^{82}Gly to Glu in the ORF-3 product are responsible for the alkali sensitivity of mutants 38154 and 18224, respectively. (From data in Fig. 2s of Hamamoto et al. 1994 and of Seto et al. 1995)

between the *Bcl*I and *Acc*I sites in ORF-1 (Fig. 1), and that the ORF-1 product is responsible for the Na^+/H^+ antiport activity that is important for the alkaliphily of strain C-125.

To identify the mutation site in the genome of mutant 38154, the nucleotide sequence of the corresponding DNA fragment was determined. Direct sequencing revealed a substitution mutation from G to A that results in a single amino acid change in the 393rd residue in the ORF-1 product (Fig. 2). We then cloned a 5.1-kb DNA fragment containing ORF-1 to ORF-4, and a restriction map of the entire cloned fragment is shown in Fig. 2. Another alkali-sensitive mutant, strain 18224, was found to have a mutation resulting in an amino acid substitution in the 82nd residue in the ORF-3 product (Seto et al. 1995). Mutant 18224 still retains the ability to control the internal pH, although it shows alkali-sensitive growth (Hashimoto et al. 1994). It appears that ORF-3 is not involved in Na^+/H^+ antiport itself but it may be involved in a regulatory process or some other function associated with ion transport.

Analyses of a *B. subtilis* homologue of the Na^+/H^+ antiporter gene of strain C-125

During the last several years, three major Na^+/H^+ antiporter genes (*nhaA*, *nhaB*, and *chaA*) have been identified in succession in *E. coli*, and several *E. coli* mutants lacking one or more of these genes have been constructed (Thelen et al. 1991; Pinner et al. 1993; Ohyama et al. 1994; Nozaki et al. 1996). Using these *E. coli* mutants as host strains, many *nhaA* and *nhaB* homologues, or genes putatively encoding a Na^+/H^+ antiporter, have been cloned from other bacteria (Pinner et al. 1992; Nakamura et al. 1994, 1996). However, the ORF-1 product of strain C-125 have not shown any similarity to known Na^+/H^+ antiporters encoded by a single gene.

Recent reports have demonstrated the existence of a novel type of cation/H^+ antiporter usually encoded by a cluster of seven genes, and our findings revealed that the Na^+/H^+ antiporter encoded by ORF-1 in strain C-125 is a

Table 1. A comparison of Sha proteins and their homologues

Strain	*Bacillus subtilis*[a]	Alkaliphilic *Bacillus* sp. C-125[b]	*Sinorhizobium (Rhizobium) meliloti*[c]	*Staphylococcus aureus*[d]
Deduced proteins	ShaA (774 aa) ShaB (143 aa) ShaC (113 aa) ShaD (493 aa)[e] ShaE (158 aa)[e] ShaF (94 aa) ShaG (124 aa)	ORF1 (804 aa) ORF2 (146 aa) ORF3 (112 aa) ORF4 (493 aa)[e]	PhaA (725 aa) PhaB (257 aa) PhaC (115 aa) PhaD (547 aa) PhaE (161 aa) PhaF (92 aa) PhaG (120 aa)	MnhA (801 aa) MnhB (142 aa) MnhC (113 aa) MnhD (498 aa) MnhE (159 aa) MnhF (97 aa) MnhG (118 aa)
Functions	Na^+/H^+ antiporter Major role in Na^+ excretion Involved in sporulation	Na^+/H^+ antiporter pH homeostasis under alkaline conditions	K^+/H^+ antiporter? Involved in infection	Na^+/H^+ antiporter

aa, amino acids
Information compiled from [a]Oudega et al. (1997); [b]Hamamoto et al. (1994); [c]Putnoky et al. (1998); [d]Hiramatsu et al. (1998)
[e]The deduced proteins newly emerged when some nucleotide sequences in the original data were corrected (Kosono, unpublished results)

member of this family. Genome sequence analysis has shown that *B. subtilis* has a *shaA* gene homologous to the ORF-1 of strain C-125 and that *shaA* exists as part of a cluster of seven genes (Oudega et al. 1997; Ito et al. 1999). Putnoky et al. (1998) identified the *pha* locus of *S. meliloti*, which contains a set of seven genes required for infection of leguminous plants by these bacteria, to establish nitrogen-fixing symbiosis. Hiramatsu et al. (1998) cloned the *mnh* locus encoding a Na^+/H^+ antiporter of *S. aureus* using an *E. coli* mutant that lacks the three major Na^+/H^+ antiporters as the cloning host. Table 1 shows a comparison of the ORF-1, ORF-2, ORF-3, and ORF-4 products of strain C-125 with the Sha proteins of *B. subtilis*, the Pha proteins of *S. meliloti*, and the Mnh proteins of *S. aureus.* The products of ORF-1 to ORF-4 correspond well to ShaA to ShaD, PhaA to PhaD, or MnhA to MnhD, respectively, in terms of protein sequence and size.

The *sha*, *pha*, and *mnh* loci each exist as a cluster of seven genes, and all seven of these genes seem to be required for the cation-transport function (Hiramatsu et al. 1998; Putnoky et al. 1998; Ito et al. 1999). Ito et al. (1999) have shown that the seven *mrp* (equivalent to *sha*) genes are transcribed as an operon. Antiporters of this family may consist of seven subunits. We have shown that the ORF-1 of strain C-125 is a necessary contributor to the Na^+/H^+ antiport, but the ORF-1 may not be necessarily the only or primary structural gene involved in the antiport. On the other hand, as mentioned earlier, the third gene of the corresponding cluster in strain C-125 seems not to be involved in ion transport directly, and the role of each of the seven genes in ion transport remains to be clarified. The homologues of the ORF-1 and ORF-4 products are also related to NuoL and NuoM of *E. coli* NADH:ubiquinone oxidoreductase (complex I), respectively (Weidner et al. 1993). However, Na^+ extrusion by Mnh or Mrp (or Sha) is severely inhibited by a proton conductor, 3-chlor carbonyl cyanide phenylhydrazon (CCCP) (Hiramatsu et al. 1998; Ito et al. 1999; Kosono, unpublished results), and it seems that these antiporters do not act as a primary pump.

To confirm whether ShaA of *B. subtilis* (homologue of ORF-1) is responsible for Na^+/H^+ antiport activity and to analyze its role in the neutralophile *B. subtilis*, we disrupted the *shaA* gene and characterized the growth phenotype as a function of Na^+ concentration and pH and the Na^+/H^+ antiport activity displayed by a *shaA*-disrupted mutant (Kosono et al. 1999). We detected decreased Na^+/H^+ antiport activity in assays using right-side-out membrane vesicles derived from the *shaA*-disrupted mutant on energization with a transmembrane proton gradient (ΔpH). The *shaA*-disrupted mutant also showed severe Na^+ sensitivity in its growth. As shown in Fig. 3, at pH 7 in LBT medium, the specific growth rate of the *shaA* mutant was identical with that of the wild type without additional NaCl but it decreased as the NaCl concentration was increased up to 200 mM, whereas that of the wild type did not change. At

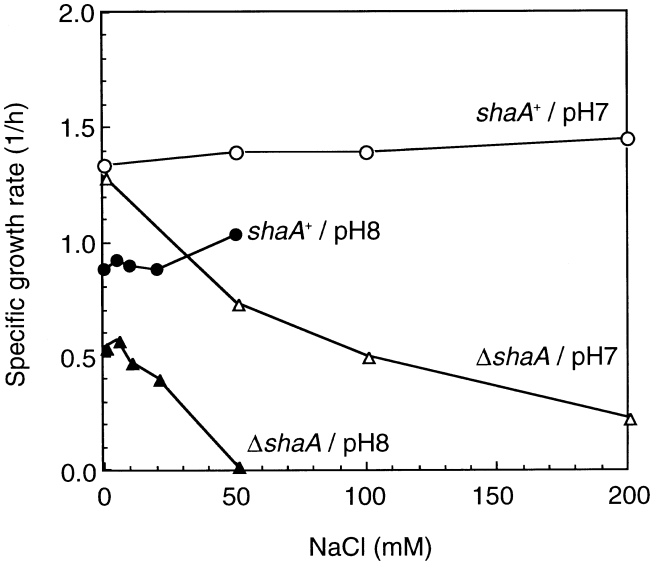

Fig. 3. Effect of NaCl on the growth of wild-type *Bacillus subtilis* 168 and that of a *shaA*-disrupted mutant. Cells of the wild type (*circles*) and the Δ*shaA* mutant (*triangles*) were grown in LBT medium medium containing various concentrations of NaCl as indicated, at pH 7 (*open symbols*) or 8 (*closed symbols*). The LBT medium contained 12 mM of endogenous contaminating Na^+. (From data in Fig. 3 of Kosono et al. 1999)

pH 8, the *shaA* mutant was more sensitive to Na^+, and it was unable to grow in the presence of 50 mM Na^+. The growth rate of the *shaA* mutant was lower than that of the wild type without additional NaCl; this is probably because of endogenous contaminating Na^+ (12 mM) in the LBT medium, and the growth rate at pH 8 was identical between the wild type and the mutant when endogenous Na^+ was limited less than 1 mM (Kosono et al. 1999). Considering that in *E. coli* loss of all three major antiporters causes the same degree of Na^+ sensitivity as that seen in the case of the *shaA* mutant (Sakuma et al. 1998), we consider that the Na^+/H^+ antiporter ShaA plays a dominant role in the extrusion of cytotoxic Na^+ in *B. subtilis.*

Antiporters of this family show a variety of characteristics and functions. It has been suggested that the *pha* locus in *S. meliloti* may encode a K^+/H^+ antiporter because *pha* mutants show sensitivity to K^+, but not Na^+, in their growth and they are deficient in diethanolamine-induced K^+ efflux (Putnoky et al. 1998). The function of the Pha system seems to be required for adaptation of these bacteria to the altered ionic milieu inside the infection thread, but the mechanism remains unclear (Putnoky et al. 1998). Ito et al. (1999) have reported that the *mrp* genes (or *sha*) are involved in pH homeostasis and cholate resistance as well as Na^+ resistance. They suggest that the *mrp* locus is related to both Na^+- and K^+-dependent pH homeostasis and may also have some K^+/H^+ antiporter capacity (Ito et al. 1999). We have more recently shown that the function of the Sha system is required for initiation of sporulation in *B. subtilis* (Kosono et al. 2000). Fine control of cytoplasmic ion levels including H^+, Na^+, and K^+ may be important for cellular processes or functions such as sporulation or infection besides pH homeostasis, and antiporters belonging to this family seem to play a predominant role in such ionic regulation. More recently, DNA regions homologous to the *sha* cluster have been found in the genome of another alkaliphilic *Bacillus firmus* OF4 (accession no. AF097740), and also in radiation-resistant *Deinococcus radiodurans* (AE001941), the purple nonsulfur bacterium *Rhodobacter capsulatus* (AF010496), and thermophilic *Thermotoga maritima* (AE001778). It seem that this gene family is widely distributed, and it will be interesting to clarify the function of each corresponding cluster.

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