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NhaR, a Protein Homologous to a Family of Bacterial Regulatory Proteins (LysR), Regulates *nha*A, the Sodium Proton Antiporter Gene in *Escherichia coli**

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On the basis of protein homology, *nha*R has previously been shown to belong to a large family of regulatory proteins, the LysR family (Henikoff, S., Haughn, G. W., Calvo, J. M., and Wallace, J. C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 6602–6606). In this work we show that *nha*R is a regulator of *nha*A, a gene encoding a Na^+/H^+ antiporter in *Escherichia coli*. Multicopy plasmid bearing *nha*R enhances the Na^+-dependent induction of a chromosomal *nha*A′-′*lac*Z fusion. Extracts derived from cells overexpressing *nha*R exhibit specific DNA binding capacity to the upstream sequences of *nha*A. Construction of an *nha*R deletion mutant (OR100) shows that *nha*R is required in addition to *nha*A to tolerate the extreme conditions under which *nha*A is indispensable. Whereas OR100 grows like the wild type at neutral pH even at high Na^+ concentrations (700 mM), it becomes much more sensitive to Na^+ (>300 mM) at pH 8.5; furthermore, OR100 is more sensitive to Li^+ (100 mM) than the wild type. Nevertheless, the phenotype of OR100, which is more resistant to Na^+, Li^+, and alkaline pH than a Δ*nha*A strain (NM81), implies that the regulation exerted by *nha*R is not complete and that some expression of *nha*A exists in OR100. Accordingly, the effect of *nha*R in cells is dependent on the level of *nha*A. OR200, a *nha*A and *nha*R deletion mutant, has the same phenotype as NM81. Multicopy plasmid bearing *nha*R does not change the phenotype of either OR200 or NM81. On the other hand, multicopy *nha*A renders the cells Li^+- and Na^+-resistant even without *nha*R.

All living cells maintain a sodium concentration gradient directed inward and a constant intracellular pH at around neutrality (Padan *et al.*, 1981; Roos and Boron, 1981). Hence, all cells have Na^+ extrusion system(s) and homeostatic mechanisms controlling the H^+ and possibly also the Na^+ circulation across the cytoplasmic membrane (Bakker, 1990; Booth, 1985; Boron, 1983; Haussinger, 1988; Padan *et al.*, 1981; Slonczewski *et al.*, 1981; Castle *et al.*, 1986). Sodium proton antiporters have been suggested to play a major role in these homeostatic mechanisms both in eukaryotes and prokaryotes (Padan *et al.*, 1981, 1989; Pouyssegur *et al.*, 1988).

A gene (*nha*A; previously named *ant*) encoding a sodium proton antiporter in *Escherichia coli* has been mapped at 0.1 min on the *E. coli* chromosome (Goldberg *et al.*, 1987; Karpel *et al.*, 1988), cloned (Goldberg *et al.*, 1987), sequenced (Karpel *et al.*, 1988), and shown to encode a membrane protein of 41,000 Da (Taglicht *et al.*, 1991). The NhaA protein has recently been purified, reconstituted in proteoliposomes, and shown to exhibit Na^+/H^+ antiporter activity (Taglicht *et al.*, 1991).

The role of *nha*A in cell physiology has been studied by constructing a Δ*nha*A mutant and comparing its phenotype to that of the wild type (Padan *et al.*, 1989). It was found that *nha*A is indispensable for growth in the presence of Li^+ (100 mM) and Na^+ (0.7 M, at pH 6.8). It was also found that the Na^+ sensitivity of the mutant increases with pH so that at pH 8.6, 0.1 M Na^+ is inhibitory. Hence, *nha*A is also required for growth at alkaline pH in the presence of Na^+.

Study of the Na^+/H^+ antiporter activity of membrane vesicles isolated from the Δ*nha*A mutant as compared to membrane vesicles isolated from the wild type revealed a new specific Na^+/H^+ antiporter activity additional to the *nha*A system (Padan *et al.*, 1989). The new system (designated *nha*B) differs from *nha*A in both ion specificity and pH sensitivity. Whereas the K_m values for Na^+ in the two systems are similar (between 0.25 and 0.6 mM), *nha*A has a 10-fold lower K_m for Li^+ (0.06 mM) as compared to *nha*B. The NhaA activity is highly dependent on pH, increasing upon alkalinization, whereas NhaB is totally independent of pH.

The involvement of at least two genes in the Na^+/H^+ antiporter activity of the cell suggests that understanding of the regulation of these systems is essential for delineation of their role in homeostasis of the cell. To study regulation of expression of *nha*A, we have constructed an *E. coli* strain (RK33Z) carrying, instead of *nha*A, a chromosomal protein fusion of *nha*A and *lac*Z (Karpel *et al.*, 1991). Monitoring β-galactosidase activity of this strain under various growth conditions showed that both Na^+ and Li^+ induce *nha*A and that alkaline pH dramatically increases the inducing effect of the ions.

The inducibility of *nha*A raises the possibility of involvement of regulatory gene(s) in the expression of *nha*A. An open reading frame capable of encoding a 28-kDa polypeptide of unknown function, which we now designate *nha*R, has been previously identified downstream of *nha*A (Mackie and Parsons, 1982; Mackie, 1986; Karpel *et al.*, 1988) and designated *ant*O (Henikoff *et al.*, 1988).

In addition to its proximity to *nha*A, *nha*R appears to be related to the antiporter gene. In the DNA sequence upstream of *nha*R separating it from *nha*A, there are no conspicuous consensus sequences of promoters. When the T7 or *tac* promoters was cloned upstream of *nha*A in a plasmid bearing an insert containing both *nha*A and *nha*R, both open reading

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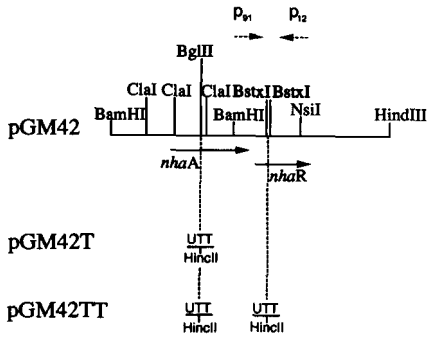

FIG. 1. **Inserts of plasmids used.** *UTT*, universal translation terminator (Karpel *et al.*, 1988). P_{91} and P_{12} are the primers used to construct plasmid pBSI1 (see "Materials and Methods").

frames were expressed from the foreign promoters (Karpel *et al.*, 1988).[1] Furthermore, on the basis of protein sequence homology, NhaR (AntO) has been included in a large family of regulatory proteins, the LysR family. Within the N terminus of all these proteins, there is a conserved helix-turn-helix motif implicated in DNA binding (Henikoff *et al.*, 1988).

To determine whether *nha*R is a regulatory gene of *nha*A, we tested the effect of deletion as well as the dose effect of the gene on expression of *nha*A. This study shows that *nha*R is a positive regulator of *nha*A that acts *in trans* and requires Na^+ for its activity.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions—The bacterial strains used in this study are *E. coli* K12 derivatives. TA15 is *mel*BLid, *nha*A$^+$, Δ*lac*ZY (Goldberg *et al.*, 1987). NM81 is *mel*BLid, Δ*nha*A1, *kan*^{R}, Δ*lac*ZY, *thr*1 (Padan *et al.*, 1989). JC7623 is *rec*B21, *rec*C22, *sbc*B15, *thr*1 (Winans *et al.*, 1985). OR1 and OR2 are JC7623 derivatives carrying Δ*nha*R1, *kan*^{R} and Δ*nha*A4Δ*nha*R2, *kan*^{R}, respectively (this work). OR100 and OR200 are TA15 derivatives carrying Δ*nha*R1, *kan*^{R} and Δ*nha*A4Δ*nha*R2, *kan*^{R} transduced by P1 from OR1 and OR2, respectively (this work). RK33Z is *mel*BLid, Δ*nha*A3, *kan*^{R}, Δ*lac*ZY, φ(*nha*A′-′*lac*Z)1(hyb) (Karpel *et al.*, 1991). Cells were grown in modified L broth, in which NaCl was replaced by KCl (87 mM, pH 7.5). When different pH values were compared, this medium was supplemented with 60 mM bis-tris,[2] and the pH was titrated with HCl. Cells were also grown in minimal medium A without sodium citrate (Davies and Mingioli, 1950) or in minimal medium MTC (Padan *et al.*, 1989). Thiamine (2.5 μg/ml) was added to all minimal media. Carbon source was 10 mM melibiose or 0.5% glycerol as indicated. When required, threonine (0.1 mg/ml) was added. For plates, 1.6% agar was used. Antibiotics were 100 μg/ml ampicillin and/or 50 μg/ml kanamycin. Resistance to Li^+ was tested by growth (for 36–48 h) on solidified minimal medium A plates containing melibiose as carbon source and 100 mM LiCl as compared with growth on similar medium containing 100 mM NaCl instead of LiCl. Resistance to Na^+ was tested by growth (for 24–48 h) on L broth plates containing 0.7 M NaCl (pH 7.5) as compared with growth on similar medium containing 0.7 M KCl instead of NaCl.

Plasmids—Plasmids used in this work are described in Fig. 1. pGM42 is a pBR322 derivative (Karpel *et al.*, 1988). pGM42T was obtained by introducing a universal translation terminator (described by Karpel *et al.* (1988)) into the *Bgl*II site of pGM42. This plasmid therefore bears an inactive *nha*A. pGM42TT was obtained by exchanging the *Bst*XI-*Bst*XI (130-bp) fragment of pGM42T with the universal translation terminator. Therefore, both *nha*A and *nha*R are inactive in this plasmid. pBSI1 is a pBluescript KS$^+$ derivative. For its construction, a chromosomal fragment was amplified by the polymerase chain reaction using chromosomal DNA isolated (Gillen *et al.*, 1981) from TA15 and primers P_{91} (AACTGGCGCGTCTGCCTG) and P_{12} (CACATTGTCGACGCGACCAATTTCTA) (see Fig. 1). The mutation (G) in P_{12} introduces an *Sal*I site (underlined); therefore, the amplified fragment that contains the chromosomal *Bam*HI site and the new *Sal*I site was digested with these enzymes, and the *Bam*HI-*Sal*I fragment was ligated into *Bam*HI/*Sal*I-digested pBluescript KS$^+$. pDT2, pKR323, pKR225, and pGM36 were described by Karpel *et al.* (1988). pGP1-2 and pT7-5 were described by Tabor and Richardson (1985). pOR1 and pOR2 were constructed from pGM42. In the former, the *Bst*XI-*Bst*XI (130-bp) fragment and, in the latter, the *Bgl*II-*Nsi*I (1524-bp) fragment were exchanged with a kanamycin resistance gene derived from pUC71K (Ruther, 1980). The inserts of pOR1 and pOR2 are identical to the *Bam*HI-*Hin*dIII (5130-bp) and *Bam*HI-*Hin*dIII (3436-bp) fragments of OR100 and OR200 depicted in Fig. 3, respectively.

Amplification of Bacterial Chromosomal DNA by Polymerase Chain Reaction—The polymerase chain reaction was carried out in a 100-μl final volume containing 1 μg of chromosomal DNA, 50 mM KCl, 10 mM Tris-Cl (pH 9, 25 °C), 1.5 mM magnesium chloride, 0.1% (w/v) gelatin, 1% Triton X-100, a 200 μM concentration of each of the deoxynucleotide triphosphates, 50 pmol of oligomeric primers, and 2.5 units of *T. aquaticus* DNA polymerase (Promega Biotec). The reaction mixture was overlaid with 100 μl of light mineral oil (Sigma) and subjected to 40 cycles of denaturation (1 min, 94 °C), annealing (45 s, 58 °C), and extension (3 min, 72 °C) using a DNA programable thermal controller (M. J. Research Inc., Model PTC 100). The amplification products were analyzed on 0.7% agarose.

DNA Sequencing—Plasmid pBSI1 was utilized to obtain various size deletions of *nha*R by the Erase-a-base kit (Promega Biotec) using *Kpn*I and *Xho*I to generate 3′- and 5′-overhangs, respectively. The different plasmids obtained were sequenced using the Sequenase kit (United States Biochemical Corp.). Reactions were primed with the universal primer (M13 reverse sequencing primer (−24)) of pBluescript KS$^+$.

Induction of nhaA′-′lacZ—RK33Z cells, transformed with various plasmids as indicated, were induced with Na^+ (100 mM) or Li^+ (50 mM); and the β-galactosidase activity of the cells was determined as described by Karpel *et al.* (1991).

DNA-DNA Hybridization—Chromosomal DNA was prepared by the method of Gillen *et al.* (1981). Restriction endonuclease digests of the DNA were resolved on horizontal 1% agarose gels and transferred to nitrocellulose membranes by a modification of the procedure of Southern (1975). The filters were hybridized with probes labeled with [α-^{32}P]dCTP by the multiprime method (Amersham Corp.).

Labeling of nhaR—For expression of *nha*R, the T7 expression system was used (Tabor and Richardson, 1985). pDT2 or pT7-5 was transformed into TA15/pGP1-2, and the transformants were pulse-labeled with [^{35}S]methionine (10 μCi, 1350 Ci/nmol) in the presence of rifampicin. The labeled cells were disrupted by sonication, and the cell-free suspension was centrifuged at 100,000 × *g* for 1 h. Proteins of the membranes and the supernatant were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography as previously described (Karpel *et al.*, 1988).

DNA Binding—The DNA probe was prepared by restriction of pGM42 with *Cla*I and purification of the 410-bp fragment which was then end-labeled with ^{32}P using the Klenow fragment of DNA polymerase. This probe contains 396 bp upstream and 14 bp downstream of the initiation codon of *nha*A (Karpel *et al.*, 1991). To prepare cell extracts, cells were grown in 500 ml of modified L broth (OR200 at 37 °C and TA15/pGP1-2/pT7-5 and TA15/pGP1-2/pDT2 at 30 °C) to A_{600} = 0.4–0.6. To overexpress *nha*R by the T7 promoter (Tabor and Richardson, 1985), cells carrying the plasmids were shifted for 25 min to 42 °C, rifampicin (100 μg/ml) added, and the cells were shifted back to 37 °C for another 90 min. All cells were harvested by centrifugation; resuspended in 6 ml of buffer containing 20 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM EDTA, and 10% (w/v) glycerol; and broken in a French press (American Instruments, Silver Spring, MD) at 18,000 p.s.i. Intact cells were separated by centrifugation (10,000 × *g*) for 10 min at 4 °C, and membranes by ultracentrifugation (100,000 × *g*) for 90 min at 4 °C. The supernatant was dialyzed overnight at 4 °C in buffer containing 10 mM Hepes (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 50% glycerol. The cell-free extract was stored at −70 °C.

DNA binding assay was conducted according to Nachaliel *et al.* (1989). About 3 ng of the DNA probe were incubated for 60 min at 25 °C with 0.12 μg of protein from the crude extracts in a 10-μl total

[1] D. Taglicht, E. Padan, and S. Schuldiner, unpublished data.

[2] The abbreviations used are: bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s); kb, kilobase(s).

volume of a reaction mixture containing 50 mM Tris-HCl (pH 7.4), 70 mM KCl, 10 mM β-mercaptoethanol, 1 mM EDTA, and 3 μg of poly(dI·dC) (Boehringer Mannheim). The reaction was stopped by adding 4 μl of stopping solution containing 90% formamide, 10 mM NaOH, 0.2% xylene cyanol, and 0.2% bromphenol blue. The DNA-protein complex was resolved on 5% acrylamide gel containing 0.5% bisacrylamide, 22.5 mM Tris borate and 0.5 mM EDTA. The gel was dried and autoradiographed.

Cell Protein—Cell protein was determined according to Bradford (1976).

RESULTS

Sequence of nhaR—On the basis of protein sequence homology, NhaR (AntO) has been suggested to belong to the LysR family of regulatory proteins (Henikoff *et al.*, 1988). While considering the possibility that *nha*R (which maps downstream of *nha*A) is a regulator of *nha*A, we re-examined the published DNA sequence of *nha*R (Mackie, 1986) and restriction maps overlapping its chromosomal location (Mackie, 1980; Kohara *et al.*, 1987) and found a discrepancy between these data. The *Pst*I site predicted from the sequence is absent from the two independently published restriction maps. Furthermore, a search for DNA homology between *nha*R and DNA of the data bank showed, at the published C terminus of *nha*R, a stretch of 14 bases identical to that of M13. Therefore, we have resequenced *nha*R.

Whereas most of the published DNA sequence of *nha*R has been confirmed, the differences found in the 3′-end of *nha*R are shown in Fig. 2. As a result of the differences, the predicted NhaR is now 301 amino acids long (M_r = 34,300), which is slightly larger than previously suggested (Mackie, 1986).

In accordance with other members of the LysR family (Henikoff, 1988), the N terminus shows conservation including the helix-turn-helix domain, and the homology drops off toward the C terminus. A hydropathic evaluation of the amino acid sequence according to the method of Engelman *et al.* (1986) reveals a largely hydrophilic protein with two putative transmembrane segments (starting at amino acids 161 and 227). We have expressed NhaR using the T7 expression system as developed by Tabor and Richardson (1985). A DNA fragment containing *nha*R was fused downstream of the T7 promoter, and plasmid pDT2 was obtained (Karpel *et al.*, 1988). NhaR was specifically labeled in the presence of rifampicin, and ~40% of the label was found in the membrane fraction of the cells even after extensive washes (data not shown). This distribution of NhaR between the membrane and the cytoplasm is not self-evident and is under more detailed study.

Construction of nhaR Deletion Mutants—To elucidate the role of *nha*R in *E. coli*, we have chosen to disrupt chromosomal *nha*R and to compare the phenotype of Δ*nha*R to that of wild-type cells. For this purpose, we have constructed plasmids pOR1 and pOR2. While leaving *nha*A intact in pOR1, *nha*R was disrupted by exchanging its *Bst*XI-*Bst*XI fragment with the kanamycin gene. In pOR2, almost all of *nha*A and *nha*R have been exchanged with the kanamycin gene. In both plasmids, the disrupted genes are flanked with sufficiently long chromosomal sequences allowing homologous recombination, exchanging the chromosomal wild-type genes with the plasmid mutant genes, following transformation of the linearized plasmids into the *rec*BC*sbc*B mutant JC7623 (Winans *et al.*, 1985). The recombinants were selected as $Kan^R Amp^S$ colonies on modified L broth plates.

Recombinants OR1 and OR2 were obtained from plasmids pOR1 and pOR2, respectively. Since all our previous physiological studies have been conducted in strain TA15, the mutations were transduced from OR1 and OR2 into TA15 by P1 transduction, selecting for Kan^R colonies yielding OR100 and OR200 strains, respectively (Fig. 3*A*).

To verify that the transductants contain the respective mutations, we used hybridization to look for the presence of different DNA sequences in the DNAs of OR100 and OR200 as compared with the DNA of the wild type. Examples of these studies are shown in Fig. 3 (*B* and *C*). When the kanamycin gene was used as a DNA probe, hybridization was observed with the DNAs, of OR100 and NM81, but not with that of TA15 (Fig. 3*B*). As expected from the restriction maps of NM81 (Padan *et al.*, 1989) and OR100 (Fig. 3*A*), two *Hin*dIII-*Hin*dIII fragments hybridized in each case: 1.28- and 3.25-kb fragments in NM81 and 1.04- and 4.10-kb fragments in OR100. When the *Bgl*II-*Nsi*I (1524-bp) fragment of pGM42 was used as a DNA probe, hybridization was observed with TA15 (1.9- and 5.2-kb fragments), but not with OR200 (Fig. 3*C*). We therefore conclude that OR100 indeed harbors, in its chromosome, the deletion and *kan* insertion in *nha*R and that OR200 contains only the chromosomal N terminus of *nha*A and the C terminus of *nha*R, whereas the in-between sequences have been exchanged by *kan*. These results also show that like *nha*A, *nha*R is present in only one copy in the wild-type chromosome.

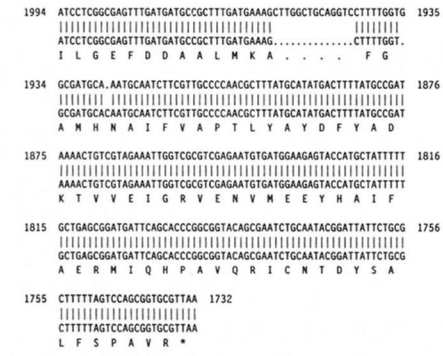

FIG. 2. **Sequence of 3′-end of *nha*R.** The correct sequence of the 3′-end of *nha*R (*lower row*) is shown in comparison to the previously published sequence (*upper row*) (Mackie, 1986). The predicted C terminus of the NhaR protein is also shown.

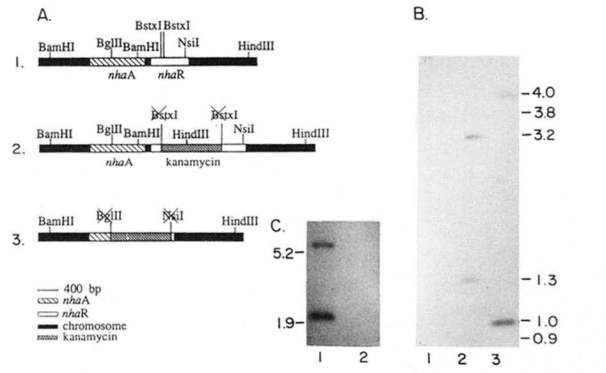

FIG. 3. **Construction of mutants OR100 and OR200 impaired in *nha*R.** *A*, for construction of the mutants, JC7623 was transformed with linearized plasmid pOR1 or pOR2, and recombinants OR1 and OR2 were obtained, respectively, by selection for Kan^R and scoring for Amp^S. Mutants OR100 and OR200 were obtained by transducing the mutations of OR1 and OR2 into TA15 by P1. The relevant parts of the wild-type chromosome (*row 1*) and those of mutants OR100 (*row 2*) and OR200 (*row 3*) are shown. *B* and *C*, Southern blots of restriction endonuclease digests of OR100 and OR200, respectively. Fragment sizes are given in kilobases. The probe used in *B* was an isolated *kan*, and the DNA was digested with *Hin*dIII. *Lane 1*, TA15; *lane 2*, NM81; *lane 3*, OR100. The probe used in *C* was a *Bgl*II-*Nsi*I (1524-bp) fragment derived from pGM42 (Fig. 1), and the DNA was digested with *Bam*HI. *Lane 1*, OR200; *lane 2*, TA15.

Growth Phenotype of Inactive nhaR Mutants—The growth phenotype of the double mutant (OR200 which lacks both *nha*A and *nha*R) is identical to the previously described phenotype of Δ*nha*A (NM81) (Padan *et al.*, 1989). Thus, mutants grow in modified L broth with no added Na^+ at the same growth rate as the wild type (doubling time, 30–35 min), and changing the pH of the medium between 6.8 to 8.5 has no effect on their growth. As opposed to the wild type, both mutants are similarly sensitive to NaCl, and pH markedly affects this sensitivity. Thus, at pH 6.8, 0.7 M NaCl is inhibitory, whereas at pH 8.5, 0.1 M NaCl is enough to completely inhibit growth of these strains (Padan *et al.*, 1989; data not shown). Both mutants are also much more sensitive to LiCl than the wild type. Addition of 100 mM LiCl to modified L broth (pH 7.5) hardly affects the growth rate of the wild type, whereas the mutants do not grow at all under these conditions (Fig. 4*A*). We conclude that Δ*nha*R does not aggravate or relieve the Li^+, Na^+, and pH sensitivity of Δ*nha*A.

Similar to OR200 and NM81, the single mutant (OR100 (Δ*nha*R), which lacks *nha*R, but contains chromosomal *nha*A) (Fig. 3) grows on modified L broth (pH 7.5) when no Na^+ is added at a growth rate similar to that of the wild type. OR100 is as resistant to Na^+ as the wild type as long as the medium pH is kept at or below 7.5. It grows like the wild type on modified L broth (pH 7.5) even in the presence of 0.7 M NaCl (doubling time, 45–50 min), a concentration that inhibits the growth of NM81 and OR200. These results show that the presence of a single copy of *nha*A even without *nha*R is sufficient to render the cells resistant to Na^+ at this pH range and that the effect of *nha*R (if any) is not essential under these conditions. Accordingly, transformation of Δ*nha*R with the pGM36 or pGM42 multicopy plasmid bearing both *nha*A and *nha*R (Karpel *et al.*, 1988) does not change the growth phenotype with respect to Na^+ at the neutral pH range.

However, when the medium (minimal medium MTC) pH was increased to pH 8.5, but the doubling time of the wild type was only slightly prolonged, even in the presence of 0.4 M NaCl, the doubling time of OR100 progressively increased with the Na^+ load. At 0.4 M NaCl, OR100 practically stopped growing at this pH (Fig. 4*B*). These results show that, pertaining to Na^+ tolerance, the phenotype of Δ*nha*R is only revealed at alkaline pH. We therefore conclude that, whereas *nha*A is required for Na^+ tolerance of *E. coli* over the entire pH range of growth (Padan *et al.*, 1989), *nha*R is indispensable for adaptation to high salinity only at the alkaline pH range.

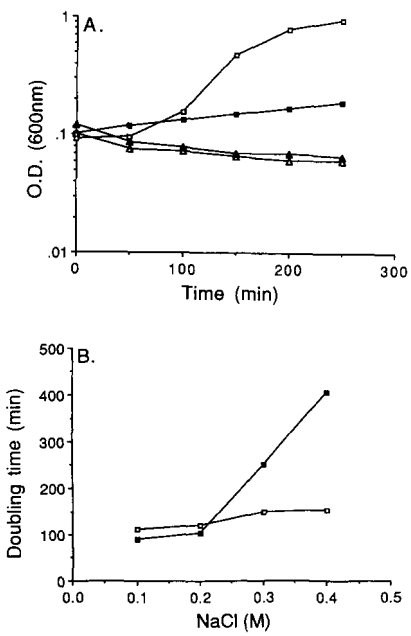

FIG. 4. **Growth phenotypes of OR100 and OR200.** *A*, cells were grown in modified L broth (pH 7.5) in the presence of 100 mM LiCl. □, TA15; ■, OR100; △, NM81; ▲, OR200. *B*, the doubling times of TA15 (■) and OR100 (□) are shown as a function of various concentrations of NaCl in minimal medium MTC (pH 8.5).

On the other hand, although the Δ*nha*R strain (OR100) is somewhat more resistant to Na^+ ions than Δ*nha*A mutant NM81 or double mutant OR200, it is much more sensitive to LiCl than the wild type (Fig. 4*A*). When grown in modified L broth (pH 7.5) in the presence of 100 mM LiCl, the doubling time increases ~10-fold. We conclude that, with respect to LiCl, both *nha*A and *nha*R are required for challenging Li^+ toxicity even at neutral pH. Accordingly, plasmid pGM42T, which bears an active *nha*R but an inactive *nha*A, complemented mutant OR100; but pGM42TT, in which both *nha*A and *nha*R are inactive, did not (data not shown).

Effect of nhaR Is Dependent on Presence of nhaA—It is possible that the effect of *nha*R on Li^+ and Na^+ resistance is independent of and additional to that of *nha*A. This possibility is unlikely since NM81, which contains an intact *nha*R but Δ*nha*A, is as sensitive to Li^+ (Fig. 4*A*) and to Na^+ (data not shown) as OR200 (Δ*nha*AΔ*nha*R). To further rule out the possibility of an *nha*A-independent effect of *nha*R, we tested the effect of multicopy *nha*R in a Δ*nha*A background. For this purpose, pGM42T was transformed into NM81 or OR200. Neither NM81/pGM42T nor OR200/pGM42T shows increased resistance to Li^+ as compared to the nontransformed mutants. We conclude that *nha*R exerts its effect only in the presence of an intact *nha*A.

It has previously been shown that multicopy *nha*A produces the $NhaA^{up}$ phenotype; it increases the resistance of the wild type or Δ*nha*A to Li^+ (100 mM) and increases the Na^+/H^+ antiporter activity in isolated membrane vesicles from such cells (Goldberg *et al.*, 1987; Padan *et al.*, 1989). Therefore, we tested the effect of multicopy *nha*A on the phenotype of Δ*nha*R. Mutants OR100 and OR200 were transformed with pKR225, and the growth characteristics of the transformants with respect to Li^+ were studied. The results showed that OR100/pKR225 as well as OR200/pKR225 have the $NhaA^{up}$ phenotype. We therefore suggest that multicopy *nha*A not only relieves the effect of Δ*nha*R, but is also capable of producing the $NhaA^{up}$ phenotype in the absence of *nha*R.

nhaR Affects Expression of nhaA—Since the effect of multicopy *nha*A is an increase in Na^+/H^+ antiporter activity (Goldberg *et al.*, 1987), the data also suggest that *nha*R exerts its effect on single copy *nha*A via increasing the expression of *nha*A and/or enhancing activity of the NhaA protein. To test the possibility that *nha*R affects expression of *nha*A, we have used *E. coli* strain RK33Z, which contains (instead of the wild-type *nha*A) a protein fusion between *nha*A and *lac*Z as a reporter gene (*nha*A'-'*lac*Z) (Karpel *et al.*, 1991). These cells were transformed with different plasmids containing *nha*A (pKR323), *nha*R (pGM42T), both *nha*A and *nha*R (pGM42), or both genes inactivated (pGM42TT). The β-galactosidase activity of the transformants was tested under different growth conditions with respect to Na^+ and Li^+ (Fig. 5).

As previously observed, when RK33Z is grown without addition of Na^+, the β-galactosidase activity is low, whereas upon addition of 100 mM NaCl, an ~5–10-fold increase in the activity is observed. Na^+ serves as an inducer since this effect is inhibited by chloramphenicol (Karpel *et al.*, 1991). The β-galactosidase activity of RK33Z/pGM42T, which bears active plasmid *nha*R but inactive *nha*A, is similar to that of the

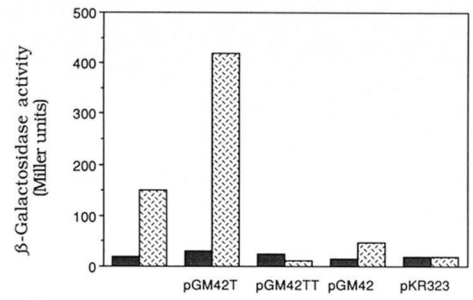

FIG. 5. **NhaR increases Na^+-dependent expression of *nhaA'-'lacZ* protein fusion.** RK33Z was transformed with pGM42T, pGM42TT, pGM42, or pKR323 as indicated. The cells were induced with Na^+ (100 mM) for 150 min. β-Galactosidase activity was determined as described (Karpel *et al.*, 1991). *Dark bars*, no Na^+; *light bars*, with Na^+.

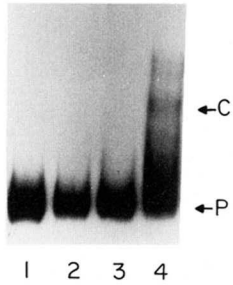

FIG. 6. **DNA binding analysis using *E. coli* extracts from cells overexpressing *nhaR*.** The DNA probe (3 ng) was incubated for 1 h at 25 °C in the absence (*lane 1*) or presence of crude extracts (0.12 μg of protein) prepared from TA15/pGP1-2/pT7-5 (*lane 2*), OR200 (*lane 3*), and TA15/pGP1-2/pDT2 (*lane 4*). The reaction mixtures were then subjected to polyacrylamide gel electrophoresis, and the dried gels were visualized by autoradiography. *P*, probe, unretarded band; *C*, gel-retarded band.

RK33Z control as long as Na^+ is not added; but, in the presence of the ion, the induction is 3–5 times higher than the effect observed in RK33Z. These results show that plasmid *nha*R affects the Na^+-dependent expression of *nha*A and that this effect is *in trans*. Accordingly, RK33Z/pGM42TT, which bears both inactive *nha*A and inactive *nha*R, had no effect on expression of *nhaA'-'lacZ*.

In contrast to the sodium-dependent effect of *nha*R on *nhaA'-'lacZ* expression in the presence of inactive *nha*A (RK33Z/pGM42T), *nha*R had no effect on expression either in the presence or absence of Na^+ when the plasmid contained an intact *nha*A (RK33Z/pGM42, RK33Z/pKR323) (Fig. 5). Although it is possible that NhaA has a direct inhibitory effect on the activity and/or expression of *nha*R, it is more plausible that the Na^+/H^+ antiporter activity of NhaA is responsible for the decreased effect of *nha*R by extruding Na^+ out of the cell. In this case, another system that extrudes Na^+ should have a similar effect. We have recently cloned *nha*B (in plasmid pEL24), an antiporter gene bearing no homology to *nha*A (Pinner *et al.*, 1992). Similar to RK33Z/pGM42 or RK33Z/pKR323, there was no induction of *nhaA'-'lacZ* in RK33Z/pEL24 (data not shown).

DNA Binding Studies of NhaR—We have previously defined two promoters of *nha*A that are both included in the *Cla*I-*Cla*I (416-bp) fragment of the upstream region of the gene (Fig. 1) (Karpel *et al.*, 1991). To determine whether *nha*R encodes a protein that binds to this DNA sequence, a gel retardation experiment was conducted with this DNA fragment as a probe and cell-free extracts of cells overexpressing NhaR (TA15/pGP1-2/pDT2) (Fig. 6). The results show that the cell-free extracts obtained from these cells contain a protein that binds to the promoter region of *nha*A. This binding is specific to the *nha*A regulatory DNA sequences since another *Cla*I-*Cla*I (520-bp) fragment of the open reading frame (Fig. 1) did not show any retardation on similar gels (data not shown). The binding is also specific to NhaR since cell-free extracts of cells lacking *nha*R (OR200) did not show gel retardation either. We could not show any effect on the mobility of the specific DNA probe with cell-free extracts obtained from wild-type cells bearing the vector plasmids without *nha*R (TA15/pGP1-2/pT7-5), suggesting that the level of NhaR is very low in these cells. Substituting 60 mM KCl in the reaction mixture with 60 mM NaCl had no effect on the binding properties of TA15/pGP1-2/pDT2 cell extracts, suggesting either that this binding is not dependent on Na^+ or that the Na^+ already existing in the reaction mixture (25 mM) is sufficient to elicit binding.

DISCUSSION

This study shows that *nha*R is a regulatory gene of *nha*A, an Na^+/H^+ antiporter gene in *E. coli* that is essential for tolerance to Na^+, Li^+, and alkaline pH in the presence of Na^+ (Padan *et al.*, 1989). Using *nhaA'-'lacZ* protein fusion, it has previously been shown that, in accordance to its physiological roles, expression of *nha*A is induced by Na^+ and Li^+ and that alkaline pH increases the magnitude of induction by the ions (Karpel *et al.*, 1991). In this work, we show that multicopy plasmid *nha*R enhances the Na^+-dependent induction of the *nhaA'-'lacZ* fusion; cells containing the fusion that carries single copy *nha*R exhibit a 5–6-fold increase in expression induced by Na^+. When the cells carry *nha*R in multidose, the increase observed with the *nha*R-bearing plasmid is ~15-fold. The fact that multicopy *nha*R has no effect without addition of Na^+ suggests that the induction by Na^+ of *nhaA'-'lacZ* involves *nha*R either directly or indirectly.

As yet, we have not directly studied the effect of *nha*R on the second Na^+/H^+ antiporter of *E. coli*.[3] However, the NhaB phenotype in the background of different doses of *nha*R suggests that *nha*R does not regulate *nha*B under the conditions tested. The phenotype of NM81, which carries single copies of both *nha*B and *nha*R, is identical to OR200 or OR200/pGM42T, strains carrying *nha*B without *nha*R and with multicopy *nha*R, respectively.

The presence of either the multicopy antiporter gene *nha*A or *nha*B inhibits the effect of Na^+ on the *nhaA'-'lacZ* fusion. Since the antiporter genes do not share significant homology, we conclude that it is the activity of the antiporter (rather than their DNA or proteins) that is responsible for eliminating the Na^+ induction. Since antiporter activity reduces intracellular Na^+, the findings suggest that intracellular (rather than extracellular) Na^+ is the inducer of *nhaA'-'lacZ* mediated by NhaR. This suggestion is in line with our interpretation (Karpel *et al.*, 1991) of the increased efficiency of induction by Na^+ at alkaline pH, a pH at which the Na^+ gradient that the cell can maintain is lower (intracellular Na^+ higher) than at neutral pH (Pan and Macnab, 1990).

NhaR is a soluble protein that exerts its effect *in trans* as shown, using plasmid *nha*R. Furthermore, extracts derived from cells overexpressing *nha*R exhibit DNA binding capacity as observed by the gel retardation assay. This DNA binding is specific to the upstream sequences of *nha*A. As yet, we could not show dependence of the DNA binding on Na^+ by addition of 60 mM NaCl to the reaction mixtures. However, the 25 mM NaCl already present in the reaction mixture may have precluded identification of this property. To test this possibility, we are currently purifying the NhaR protein.

In this work, we have deleted *nha*R from *E. coli* cells and

found that, although they contain an intact *nha*A, the Δ*nha*R cells also become sensitive to Li^+ as compared to the wild type. In modified L broth at pH 7.5, the doubling time of the wild type in the presence of 100 mM Li^+ is 50 min and that of the mutant is ~10-fold longer. These results support the suggestion that *nha*R is a regulator of *nha*A, exerting its effect not only on the *nhaA'-'lacZ* fusion protein, but also on intact *nha*A. Accordingly, Δ*nha*AΔ*nha*R has the same phenotype as Δ*nha*A, and transformation of Δ*nha*R with multicopy plasmid *nha*R (pGM42T) or *nha*A (pKR225) restores the Li^+ tolerance of the wild type.

Interestingly, Δ*nha*R is somewhat more resistant to Li^+ as compared to Δ*nha*A, suggesting that *nha*A is expressed to a certain level even without *nha*R. This constitutive expression of *nha*A can also explain the observation that deleting *nha*R has no apparent effect on the Na^+ tolerance of the cells at pH <7.5. The need of *nha*R for a maximal adaptive response to Li^+ at pH 7.5 (but not to Na^+) may suggest that a higher V_{max} for the antiporter is needed to challenge increasing Li^+ toxicity as compared to increasing salinities under these conditions. Indeed, when the dose of *nha*A is increased by transformation of cells with multicopy plasmid *nha*A, Li^+ resistance is increased above the level of the wild type (Nha^{up} phenotype), whereas tolerance to Na^+ is hardly increased above the wild-type level. Nevertheless, the requirement for *nha*R for Na^+ tolerance becomes apparent at pH 8.5. We suggest that the need for increased antiporter activity, above the constitutive level, is more pronounced under these conditions. Indeed, previous results showed that the dependence of antiporter activity for Na^+ tolerance is increased with pH. Thus, the sensitivity of NM81 (Δ*nha*A) to Na^+ is enhanced at alkaline pH (Padan *et al.*, 1989).

That NhaR is a positive regulator of *nha*A agrees with its previously documented homology to the large family of positive regulators, the LysR family. All these proteins have, at their N terminus, a conserved helix-turn-helix domain that is supposed to bind to DNA. Interestingly, several members of this large group are proteins that are involved in the response of the organism to stress. An example is OxyR, which is essential for the resistance of the organism to oxidative stress (Storz *et al.*, 1990). We suggest that *nha*R may represent a component of yet another type of stress response essential for Li^+ and Na^+ tolerance.

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