Jour. Microbiol. June 1995 p. 120-125 Vol. 33, No. 2

Regulation of the Expression of *nhaA* Gene, Coding Na^+/H^+ Antiporter A of *Escherichia coli.*

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(Received January 22, 1995/Accepted April 21, 1995)

β-galactosidase activity of *Escherichia coli* cells containing operon fusion *nhaA'-'lacZ* was monitored to study the regulation of expression of *nhaA* gene under various conditions. The expression of the fusion was enhanced only by chemicals containing Na^+ or Li^+. This $Na^+(Li^+)$-specific enhancement of β-galactosidase activity represented the increase in the rate of synthesis of β-galactosidase rather than the decrease in the breakdown rate. The induction pattern was influenced by copy numbers of the gene. Induction by Na^+ or Li^+ was concentration and time dependent, reaching maximum 5~6 fold induction after 2 hours at 0.4~0.5 M for Na^+ or at 0.25~0.35 M for Li^+. Although the expression was induced at much lower concentration of Na^+ at alkaline pH values than at neutral pH in the presence of Na^+, alkaline pH itself did not induce the expression of the fusion in the absence of Na^+. Temperature shift and growth phase of culture did not affect the level of induction.

Key words: *E. coli*, Na^+, pH regulation, fusion

Homeostasis of intracellular pH has been studied for long time in *Escherichia coli* (6, 7, 13). *E. coli* maintain internal pH at 7.6 using Na^+/H^+ antiporter (3, 4). The transiently elevated intracellular pH on exposure to alkaline environment is restored by exchanging internal Na^+ for external H^+ through the Na^+/H^+ antiporter (14, 18, 22).

Another role of the antiporter in *E. coli* is to extrude Na^+ using electrochemical gradient of H^+ establishing inward Na^+ gradient (1, 2). The gradient of Na^+ is utilized several ways. As an example, nutrients are transported into cells using the gradient.

The biochemical properties of the system can be summarized as follows; 1) it is specific with Na^+ and Li^+. 2) one Na^+ is exchanged for one H^+ at low pH and for two H^+ at alkaline pH. 3) it showes activity only at alkaline pH (1, 2).

All *nhaA* null mutants were sensitive to alkaline pH and Na^+ (Li^+), proving that this system is responsible for restoring intracellular pH and for extruding Na^+ (14, 17, 18). A gene coding Na^+/H^+ antiporter in *E. coli* (*nhaA*) has been cloned and sequenced (6, 9). The NhaA protein has been purified and reconstituted in proteoliposome (20). *In vitro* mutagenensis experiment showed that histidine is the sensor of pH (5). The evidence for two antiport systems were provided by studying the different properties of the two (17, 19).

In this paper we report the regulation of expression of the antiporter gene (*nhaA*) of *E. coli* using a strain containing *nhaA'-'lacZ* operon fusion.

Materials and Methods

Materials

O-nitrophenyl-β-D-galactoside (ONPG), 1,3-bis[tris(Hydroxymethyl)methylamino-]propane (Bis-tris propane) and most of other chemicals are reagent grade and purchased from Sigma, USA. Chemicals for culture media were purchased from Difco.

Bacteria and plasmids

A *E. coli* strain LS125 containing operon fusion *nahA'-'lacZ* was used to study the regulation of expression of *nhaA*. Construction and characterization of LS125 was reported in previous papers (17, 18). A plasmid containing *nhaA* gene (pNHA7) was constructed from a plasmid

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pBR322 in previous study (18).

Culture media

LK medium (0.638% KCl, 0.5% yeast extract and 1.0% tryptone) was used for maintenance of LS125 (19). LK medium was supplemented with various chemicals as indicated in experiments. pH values of media were adjusted using HCl and KOH. Concentrations of antibiotics (μg/ml) were 25 for kanamycin, 50 for ampicillin, and 100 for chloramphenicol.

β-galactosidase assay of fusion

One unit of specific activity of β-galactosidase was defined as amount of enzyme to produce from the substrate nano mole of 2-nitrophenol/mg of protein/minute. The cells for assay were prepared as follows. Over-night culture was diluted 100 fold in the media of choice and grown further to midlogarithmic phase of growth (OD_{540} 0.4~0.5) at 37℃ and 250 rpm. Midlog. cells were diluted 10 fold in pre-warmed media and grown further. Samples were taken at designated intervals and kept on ice. The cells of 3.0 ml were washed twice with distilled water and suspended in 1.0 ml water. β-galactosidase activity was monitored according to Miller (12).

Quantitation of protein.

Cells washed twice in distilled water were lysed in 1.0% SDS at 95℃ for 5 minutes. The amount of protein was determined according to Lowry *et al* (11).

Specificity of induction

Various chemicals were added into LK medium to 0.8 osmolarity for most chemicals to study the effect of chemicals on the expression of the fusion. The cells were grown further for two hours after the addition of chemicals.

Induction by Na^+ and Li^+

Midlog. culture was diluted in LK medium supplemented with different concentrations of Na^+ or of Li^+. The pre-determined amounts of inoculum were added to keep the culture in midlog. phase. Samples were taken after incubating for 2 hour and prepared for assay. Time course of induction was studied as follows. The midlog. cells were diluted 2 fold in LK containing 0.3 M NaCl (LiCl) and incubated further. At 20 minute intervals, half of the culture was taken for assay and pre-warmed medium of same volume was added repeatedly for 4 hours to maintain the cultures in midlog. phase.

Induction by alkaline pH

pH values of LK supplemented with bis-tris propane medium were adjusted using HCl and KOH. Bis-tris propane (60 mM) was added to increase the buffering capacity of LK medium. Predetermined amounts of inoculum was added to media of different pH values to keep the culture at midlog. phase. Samples were grown for 2 hours before β-galactosidase assay.

Induction by temperature shift

Bacteria grown at 30℃ to midlogarithmic phase were transferred 16℃ or 42℃. Cells were taken at indicated time after transfer and assayed as indicated.

Effect of growth phase on induction

Bacteria were grown in LK medium to different stages of growth. Cells were taken at different phases of growth and prepared as indicated and assayed for β-galactosidase activity.

Results

Specificity of induction

The regulation of the expression of *nhaA* gene was studied by monitoring β-galactosidase activity of a strain LS125. Various chemicals were supplemented to LK me-

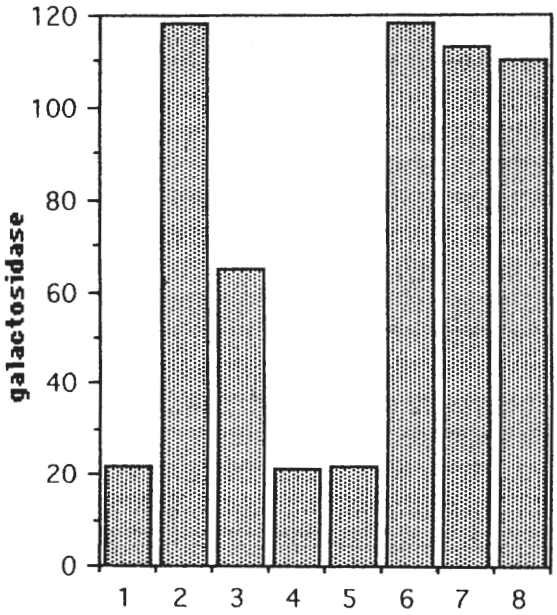

Fig. 1. The effect of various reagents on the expression of *nhaA'-'lacZ*. Cells, growing exponentially in LK (pH 7.5), were inoculated into LK (pH 7.5) supplemented with various chemicals of indicated concentrations and incubated for 120 minutes before LacZ assay. 1, without addition; 2, 0.4 M NaCl; 3, 0.2 M LiCl; 4, 0.4 M KCl; 5, 0.8 M sucrose; 6, 0.2 M Na_2HPO_4; 7, 0.4 M NaH_2PO_4; 8, 0.2 M Na_2SO_4.

Table 1. Effect of chloramphenicol on expression of *nha'-'lacZ* fusion

Time (hour) of exposure to		β-galactosidase
NaCl[a]	Chloramhenicol[a]	activity (unit)[b]
0→2		72
	0→2	21
0→2	0→2	22
0→1		72

[a] Chloramphenicol was added to 100 μg/ml and NaCl was added to 300 mM. [b] Cells of strain LS125 were grown in LK medium (pH 7.0). At the midlogarithmic phase of growth cells were exposed to various condition as indicated.

dium to study the effects on the expression of the fusion (Fig. 1). Cells grown in LK medium showed basal level of β-galactosidase activity (22 unit). This basal level of expression did not change in cells grown in LK medium supplemented with various chemicals. But addition of NaCl (0.8 osmolarity) or LiCl (0.4 osmolarity) enhanced β-galactosidase activity to 120 or 65 unit, respectively, indicating that osmotic or ionic stress is not the cause of the enhancement of the activity. All Na^+ containing compounds (Na_2PO_4, $NaHPO_4$, and Na_2SO_4) were shown to enhance the expression of the fusion to similar levels (110~120 unit), ruling out anions as the cause of the increase. This Na^+ (Li^+) specific enhancement of expression is consistent with the role of the antiporter to extrude intracellular Na^+ or Li^+.

The enhancement of β-galactosidase activity could result from the increase in the synthesis of LacZ or from the decrease in breakdown of the protein. Chloramphenicol was supplemented during the induction to clarify this ambiguity (Table 1). Addition of 300 mM of NaCl enhanced level of LacZ to 72 unit. When chloramphenicol was added simultaneously with NaCl, 300 mM NaCl did not enhance the expression of the fusion (22 unit). This result clearly indicates that the increase in activity was due to increased synthesis. When chloramphenicol was added 1 hour after the addition of NaCl, chloramphenicol did not affect the induction level of LacZ, indicating that most of the protein was synthesized during the first one hour induction and that the protein remained active for the next one hour.

Induction by Na^+ or Li^+

The effect of different concentrations of NaCl on the expression of fusion is shown on Fig. 2. The induction level steadily increased with increasing concentration of NaCl up to 0.5 M, reaching the maximum of 100 unit of at 0.4~0.5 M and decreased at higher concentrations of NaCl. The decrease of the activity may be due to

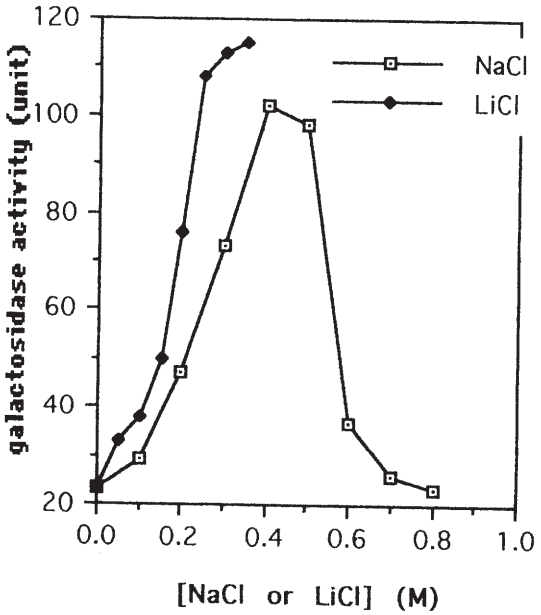

Fig. 2. The effect of concentrations of NaCl and LiCl on the expression of *nhaA'-'lacZ*. Cells, growing exponentially in LK (pH 7.0), were inoculated into LK medium (pH 7.0) supplemented with NaCl and LiCl of indicated concentrations and incubated for 2 hours before LacZ assay.

the fact that LS125, which lacks Na^+/H^+ antiporter activity, did not grow at high concentration of NaCl. Addition of LiCl also induced the expression of the fusion. At the same concentrations of ions, induction level were higher with Li^+ than with Na^+. This is consistent with the fact that Li^+ is more toxic than Na^+. The maximum enhancement of the expression was 5~6 fold (110 unit at 0.25~0.35 M). Since Li^+ is toxic to bacteria, the expression level higher than 0.4 M was not tested. Time course of induction were studied for Na^+ and Li^+ (Fig. 3). After adding NaCl to 0.3 M, level of induction increased 47, 75, and 95 unit at 20, 40, and 60 minutes, respectively. This induction level was maintained at maximum level for 80 minutes (60~140 minutes). After 140 minutes, induction level slowly decreased and reached 80 unit at 240 minutes (Fig. 3). With 300 mM LiCl the induction levels were 85, 100, and 113 unit at 20, 40, and 60 minutes, respectively. Induction level steadily decreased to 90 unit at 240 minutes after reaching the maximum level at 60 minutes.

Effect of copy numbers of *nhaA*.

Strains LS125 and LS125 (pNHA7) were used to study copy number effect. Plasmids pNHA7 is a derivative of pBR322, whose copy numbers are known to be 15~20. A plasmid pNHA7 contains *nhaA* gene (17, 18). The levels of fusion were 70 and 12 unit for LS125 and LS125

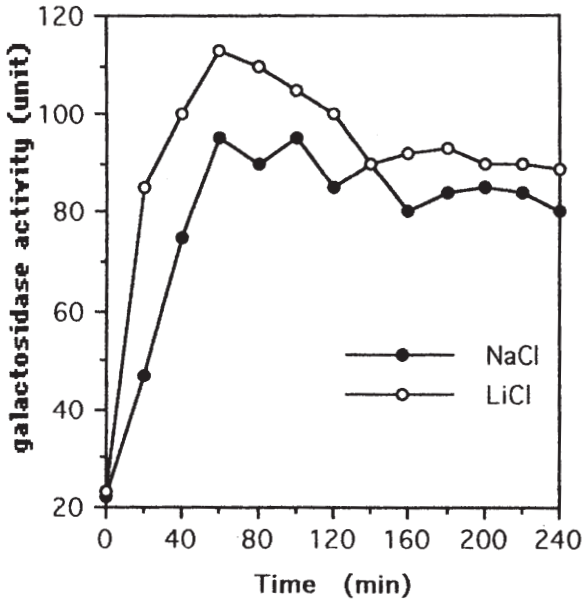

Fig. 3. Time course of induction of the expression of *nhaA'-'lacZ* by NaCl and LiCl. Cells, growing exponentially in LK (pH 7.0), were inoculated into LK medium (pH 7.0) supplemented with 300 mM NaCl (LiCl) and incubated for different time period before LacZ assay.

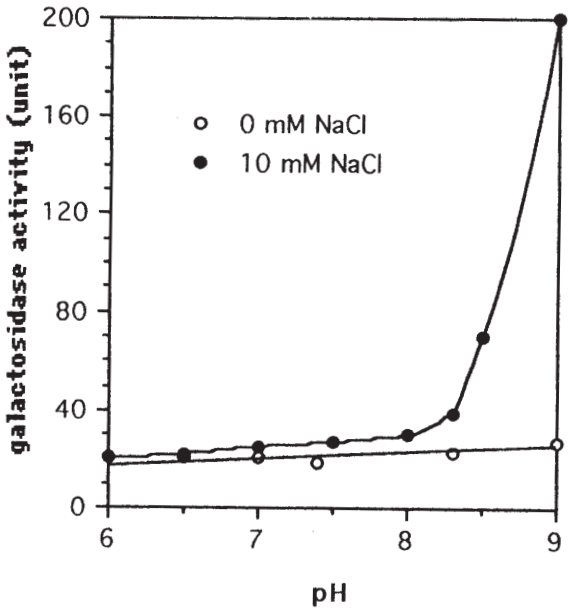

Fig. 4. Effect of pH on the expession of *nhaA'-'lacZ*. Cells, growing exponentially in LK (pH 7.4), were inoculated into LK media of indicated pH values without or with 10 mM NaCl and incubated for 2 hours before LacZ assay. The media were supplemented with 60 mM bis-tris propane.

(pNHA7), respectively. The low activity of LS125 (pNHA 7) can be explained by the titrating out the positive regulator NhaR, which enhanced the expression (16).

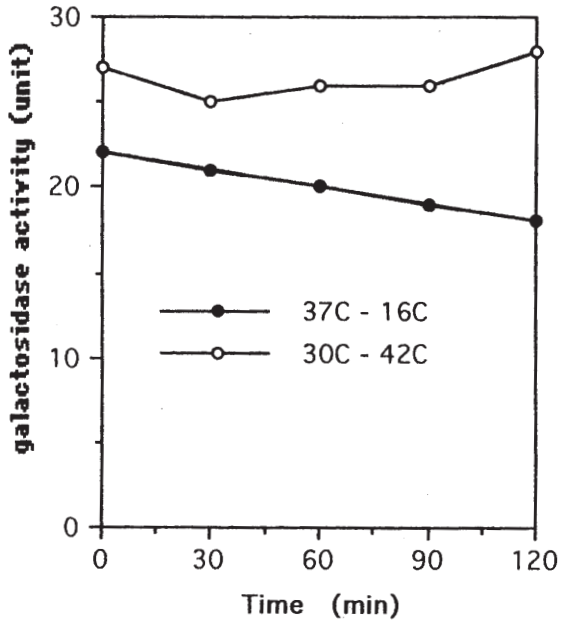

Fig. 5. The effect of temperature on the expression of *nhaA'-'lacZ*. Cells, growing exponentially at 37℃ or 30℃ in LK (pH 7.0), were inoculated into LK (pH 7.0) at 16℃ or 42℃, respectively, and incubated for different time periods before LacZ assay.

Effect of pH values

The effect of pH values on the expression of the fusion was studied (Fig. 4). Without NaCl in the media, induction levels at all pH values were similar to basal level at pH 7.0 (20, 20, 18, 23, 30 unit at pH values of 6.5, 7.0, 7.4, 8.3, and 9.0 respectively) (Fig. 4). Induction by alkalinity requires Na^+. In the presence of 10 mM NaCl the expression was maintained to basal level up to pH 8.0 (20, 22, 25, 27, and 30 unit at pH values of 6.0, 6.5, 7.0, 7.5, and 8.0, respectively). Higher pH values resulted in sharp increase in the expression (39, 70, and 200 unit at pH values of 8.3, 8.5, and 8.8, respectively) (Fig. 4). This experiment shows that alkalinity itself does not induce the expression, but potentiates the induction by low concentration of Na^+ (or Li^+) (8).

Effect of temperature

Since *nhaA* locates next to heat-shock gene *dnaK* (8), the effect of temperature was studied (Fig. 5). The shift to lower temperature (from 37℃ to 16℃) did not induce the expression (27, 25, 26, 26, and 27 unit at 30, 60, 90, and 120 minutes after temperature shift, respectively). The expression level did not either change after the shift to higher temperature (from 30℃ to 42℃) (23, 22, 20, 19, and 19 unit at 30, 60, 90, and 120 minutes after the shift, respectively). This experiment shows that the expression of *nhaA* is not controlled by temperature.

Effect of growth phase

Whether expression is influenced by growth phase, expression level is checked at different stages of growth. In absence of NaCl expression was maintained at basal level (18, 18, 20, 18, 17, and 18 unit at 0.84, 1.5, 2.28, 2.42, 2.46, and 2.57 of OD_{540} of culture, respectively). This experiment shows that expression is not influenced by growth stage.

Discussion

In present report we studied the regulation of expression of *nhaA*. Only Na^+ or Li^+-containing compounds induced the expression after exposure of cells to various chemicals (Fig. 1). The induction levels were dependent on concentration of Na^+, reaching maximum level at 0.4 M to 0.5 M Na^+ (Fig. 2). The concentration-dependent trigger may be intracellular Na^+ concentration. Since *E. coli* maintains constant concentration gradient of Na^+ across the membrane, the exposure of cells to increasing concentrations raises the intracellular concentration of Na^+. Furthermore, intracellular concentration of Na^+ becomes higher at alkaline pH values because Na^+ gradient becomes smaller at alkaline pH than at neutral pH (3,4). Higher intracellular concentration of Na^+ rather than alkalinity may be the cause of the induction. This is substantiated by the fact that alkaline pH did not induced the expression in the absence of Na^+ (8).

NhaA plays major role in restoring intracellular pH in alkaline environment and in lowering raised intracellular Na^+ in saline environment (14, 18). The induction of *nhaA* by Na^+ or the potentiation of the induction by alkalinity is quite consistent with the role of this antiporter. Na^+/H^+ antiporter activity are regulated at two different level; activation of pre-made antiporter and induction to make more protein. When internal pH values and concentrations of Na^+ are elevated, immediate response is the activation of basal antiporter. Under the continuous stress, the amount of the antipoter may be not sufficient. Later response is to make more antiporter. Since the previous study with protein fusion showed the similar results to that with operon fusion, the increased synthesis is due to the increased transcription (8). Since the fusion points are different and the difference in joint point can give rise to the different regulation, direct comparison of the results may not be good. At present we are constructing two classes of fusions with exactly the same joint points using the methods of Wilmes-Riesenberg and Wanner (21). Comparison of regulation pattern of the two classes of fusions will reveal the translational control.

Alkaline pH induced the expression of the fusion in the presence of Na^+, but not in the absence of Na^+. Since the activity of the Na^+/H^+ antiporter is required to lower the alkaline intracellular pH, the lack of induction by alkaline pH seems peculiar. Since the antiport activity is assayed in buffer containing Na^+ as subtrate, whether the antiporter is active or not in the absence of Na^+ were not studied further. Since *E. coli* is living in the environment containing sufficient amount of Na^+, the lack of induction by alkaline pH without is not a big problem for *E. coli*.

Acknowledgement

This work was supported by a grant to Sung-Yum Seo from The Ministry of Education.

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