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Sodium Chloride Induces an NhaA/NhaR-Independent Acid Sensitivity at Neutral External pH in *Escherichia coli*

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Escherichia coli **previously grown in low-salt broth, pH 7.0, produced organisms which were markedly more acid sensitive when subsequently cultured in the same broth with 200 mM or more salt (NaCl) added. Induction of acid sensitivity occurred rapidly at both 37 and 30°C, with a substantial effect within 15 min. Sensitization was partially inhibited by chloramphenicol and tetracycline and may depend on both protein synthesis-dependent and -independent physiological changes in the NaCl-induced organisms; sensitization did not result from osmotic shocking on transfer to challenge medium. Induction of acid sensitivity was affected by neither the sodium ion pore inhibitor amiloride nor the DNA synthesis inhibitor nalidixic acid; rifampin had a small effect, similar to that of chloramphenicol. Chlorides of other monovalent cations, especially Li^+ and NH_4^+, also produced sensitization to acid, although CsCl was ineffective but did not interfere with sensitization by NaCl. Other sodium salts were also active as sensitizers, as were chlorides of divalent cations, but although sucrose (but not glycerol) was a good inducer, the results were not fully in accord with triggering of induction solely by the NaCl-associated increase in osmotic pressure. Sensitization was not prevented by deletion of the *nhaA*, *nhaR*, or *nhaB* gene. Acid sensitivity of NaCl-induced cells was slightly reduced after 90 min of growth at 37°C in low-salt broth but was completely lost after 240 min. For NaCl-induced cells, acid killing in challenge media was not inhibited by amiloride. The NaCl-induced sensitization is distinct from the phenomenon of acid sensitivity induction in *E. coli* at alkaline external pH.**

Enterobacteria can be subjected to high concentrations of salt (NaCl) in a wide range of situations. In an aquatic environment, polluting organisms may enter marine and estuarine waters where the NaCl concentrations can be as high as 500 mM. Contaminating organisms which enter foods may be exposed to NaCl in those which are naturally or artificially salted or preserved in brine. Also, organisms ingested in food or water are subjected to NaCl concentrations of up to 150 mM in an animal body. The present study established that on exposure to NaCl, *Escherichia coli* rapidly becomes highly acid sensitive.

The ability to resist stress is very often increased by prior exposure to low doses of the same stress. Thus, pre-exposure to heat, alkylating agents, oxidative stress, ethanol, or starvation (2, 9, 12, 14, 23) leads to enhanced tolerance for these stresses. Responses to pH stress are of particular interest because organisms can be exposed to extremes of pH in aquatic environments, in foods, and in animal and human bodies (21), and responses to such stress may influence subsequent ability to survive and cause disease (4). Organisms switch on at least two classes of response when exposed to pH extremes. (i) Enzymes and other proteins are synthesized which may enhance growth at the exposure pH, e.g., by catalyzing the formation of components able to partially neutralize the extreme pH (5, 6, 13, 25), or there may be production of an alternative porin which is, presumably, more favorable for growth at the exposure pH (24). (ii) Induction of stress tolerance may occur, enabling the organisms to survive exposure to pH values more extreme than those which induce the response (1, 3, 4, 11, 21). Pre-exposure to one stress can sometimes alter the response to another (8, 9). One such altered response is acid sensitivity induction (ASI); incubation at alkaline external pH (pH_o) rapidly leads to profound acid sensitivity with protein synthesis-dependent and -independent sensitization components (19, 20).

Although enterobacteria are frequently exposed to high external NaCl concentrations, cytoplasmic levels of Na^+ must be kept low or growth ceases. The active extrusion mechanisms which operate under these conditions involve the NhaA and NhaB antiporters, which extrude Na^+ concomitant with H^+ influx and are dependent on proton motive force (10, 16–18). The NhaA pore is inducible by sodium ions and positively regulated at the transcriptional level by the NhaR protein. In contrast, the NhaB pore level is unaffected by the Na^+ level and its synthesis is not NhaR regulated (10, 17, 18). Since these antiporters catalyze entry of H^+ associated with Na^+ efflux, it seemed possible that the acid sensitivity of organisms briefly exposed to NaCl might involve changes in antiporter functioning.

MATERIALS AND METHODS

Bacterial strains. All of the strains used were derivatives of *E. coli* K-12. They are listed in Table 1 with their genetic characteristics and sources.

Media and growth conditions. Unless otherwise stated, organisms were grown in low-salt broth at 37°C; this broth contained 10 g of Oxoid L37 Peptone and 10 g of Oxoid L29 Lab Lemco powder per liter. After overnight growth with shaking, cultures were diluted 20- to 50-fold in fresh medium and the exponential-phase cultures which resulted after 2 to 3 h of further growth while shaken at 37°C were used for tests of acid sensitivity or induced with salts before challenge. For acid challenge, Oxoid No. 2 broth was used; this contains 10 g of

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TABLE 1. Derivatives of *E. coli* K-12 used in the present study

Strain	Genotype	Source
TA15	*melBLid rpsL ΔlacZY*	S. Schuldiner
NM81	TA15 *ΔnhaA1* Kan^r	S. Schuldiner
OR100	TA15 *ΔnhaR1* Kan^r	S. Schuldiner
OR200	TA15 *ΔnhaA4 ΔnhaR2* Kan^r	S. Schuldiner
1157	*thr leu proA2* (*ΔproA phoE gpt*) *his thi argE lacY galK xyl rpsL*	N. E. Gillies
1829	*trp*	This laboratory
MC4100	*araD139* Δ(*argF lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	M. Casadaban
PCO479	*thr leu thi pyrF codA thyA argG ilvA his lacY tonA tsx phx rpsL deoC susuvrB vtr supE glpR*	B. Lugtenberg
P678-54	*thr leu thi lacY ara xyl rpsL lamB*	H. Adler
W3110	Δ(*argF lac*)U169 *ara malB gyrA*	H. Kobayashi
RS1	Δ(*argF lac*)U169 *ara malB gyrA ΔnhaA1 nhaB*	H. Kobayashi

TABLE 2. Acid sensitivity is not induced in 300 mM aqueous saline at 37°C[a]

Induction medium	Induction time (min)	% Survival after exposure to pH_o 3.0 for 5 min
Expt 1		
None	0	20.0
NaCl-broth	5	6.3
NaCl-broth	15	1.0
NaCl-broth	30	0.01
NaCl-broth	60	0.004
Expt 2		
None	0	15.2
Aqueous saline	15	20.0
Aqueous saline	30	21.8
Aqueous saline	60	5.2

[a] Strain TA15 organisms were grown to the exponential phase in pH 7.0 low-salt broth at 37°C and, after being washed, shifted to the same medium with 300 mM NaCl added (NaCl-broth) or to 300 mM aqueous saline (pH 7.0). Acid sensitivity was assessed in No. 2 broth (see Materials and Methods) prior to the shift and at the stated intervals afterwards.

Oxoid L37 Peptone, 10 g of Oxoid L29 Lab Lemco powder, and 5 g of NaCl per liter. Nutrient agar was prepared by solidifying No. 2 broth with 2% (wt/vol) Difco Bitek Agar.

Osmolality. Osmolalities of media were measured with a Wescor 5100C vapor pressure osmometer. The instrument was calibrated against freshly made aqueous NaCl standards before use.

Acid killing. Suspensions of organisms in No. 2 broth acidified with HCl were shaken at 37°C, and samples were removed at intervals, diluted in pH 7.0 No. 2 broth, and plated on nutrient agar with incubation for 24 to 30 h. Monitoring showed that the pH of the medium did not change during challenge.

Results reported in the tables and the figure for survival after acid challenge are, unless otherwise stated, for single experiments. All tests were, however, repeated with consistent results, and some experiments were performed three or more times and the results were analyzed statistically as described by Hicks and Rowbury (7).

RESULTS

Rapid induction of acid sensitivity. Acid sensitivity was rapidly gained in NaCl-supplemented broth at 37°C. There was only slight sensitization after 5 min but a marked effect after 15 min, and the response was almost complete at 30 min (Table 2). Analysis of data for 11 experiments showed that uninduced cells gave survival percentages of 16.6% ± 2.4% (standard error) and 11.9% ± 2.1% after exposure to a pH_o of 3.0 for 5 and 7 min. In contrast, cells induced with NaCl for 30 min showed survival percentages of only 0.08% ± 0.025% and 0.04% ± 0.010% after the same challenge periods. Induction of acid sensitivity by NaCl was also rapid at 30°C, with marked sensitization within 15 min.

With 30 min of induction, NaCl produced very little effect when added at 50 or 100 mM; sensitization was marked at 150 to 200 mM and almost fully complete at 300 mM (Fig. 1 and Table 3). Neither CsCl (Table 3) nor KCl (at 300 mM) reduced sensitization by NaCl.

Other *E. coli* K-12 strains were tested for sensitization by NaCl. In a representative experiment, strain 1829 grown in low-salt broth showed 5.4% survival after 5 min at pH_o 3.0 but cells pregrown for 30 min in such broth plus 300 mM NaCl gave a survival percentage of only 0.08% under the same challenge conditions; strain W3110 showed a similar effect, as did strains P678-54 and PC0479. In contrast, prior to NaCl exposure, strain 1157 organisms showed 2.0% survival with a 7-min challenge at pH_o 3.0, whereas after exposure to NaCl for 30 min, survival was essentially unaffected, i.e., 2.2% under the same challenge conditions. Similarly, strain MC4100 was not sensitized by NaCl, with low-salt-exposed cells showing 2.0% survival after 5 min at pH_o 3.0 and those pregrown with NaCl

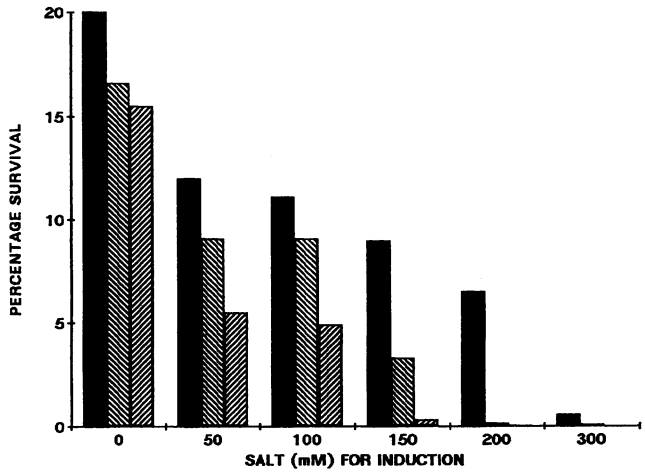

FIG. 1. NaCl concentration and acid sensitivity. Strain TA15 organisms were grown to the exponential phase in low-salt broth at 37°C and then shifted to the same medium with the concentrations of added NaCl shown. After 30 min in the new medium, acid sensitivity was assessed (in pH 3.0 No. 2 broth for 3.5, 5.0, and 7.0 min). The osmolalities of the broths were as follows: low-salt broth, 135 mosmol/kg; with 50 mM NaCl, 230 mosmol/kg; with 100 mM NaCl, 320 mosmol/kg; with 150 mM NaCl, 410 mosmol/kg; with 200 mM NaCl, 505 mosmol/kg; with 300 mM NaCl, 680 mosmol/kg. Key: ■, acid for 3.5 min; ▨, acid for 5.0 min; ▨, acid for 7.0 min.

TABLE 3. Specificity of induction of acid sensitivity[a]

Inducer (concn [mM])	Osmolality of induction medium (mosmol/kg)	% Survival after pH_o 3.0 for 5 min
None	125	13.3
NaCl (300)	675	0.07
NaCl (600)	1,260	0.001
LiCl (300)	680	0.2
NH_4Cl (300)	645	0.02
KCl (300)	665	4.2
KCl (600)	1,222	0.07
RbCl (300)	655	2.0
CsCl (300)	645	36.5
NaCl (300) + CsCl (300)	1,225	0.08
NaBr (100)	340	5.8
NaBr (200)	495	0.02
NaBr (300)	675	0.01
$NaNO_3$ (300)	648	0.01
Na_2SO_4 (100)	345	0.5
Na_2SO_4 (200)	565	0.01
Na_2SO_4 (300)	777	0.03
$MgCl_2$ (200)	645	0.06
$MgCl_2$ (300)	933	0.01
$CaCl_2$ (200)	475[b]	21.4
$CaCl_2$ (300)	625[b]	1.3
Sucrose (300)	456	11.5
Sucrose (600)	824	0.1
Glycerol (600)	752	8.3

[a] Strain TA15 was grown to the exponential phase at 37°C in low-salt broth and shifted to the same broth with the indicated inducers added. Osmolalities of broths were measured as described in Materials and Methods. After induction for 30 min, organisms were harvested and tested for acid sensitivity in acidified No. 2 broth. With a 300 mM inducing concentration, the values for three experiments showed the survival (after 5 min at pH_o 3.0) of NaCl-induced cells to be 0.05% ± a standard error of 0.01%. The survival values of cells induced with KCl and RbCl (2.8% ± 0.8% and 2.1% ± 0.7%, respectively) were significantly greater (99% confidence) than these but were significantly less than that of uninduced cells (19.4% ± 3.1%). Values for CsCl-induced cells (mean survival value, 21.7% ± 8.6%) were not significantly different from those of uninduced cells.

[b] Osmolalities of broths containing $CaCl_2$ were low, especially at 300 mM (the expected osmolality for 200 mM $CaCl_2$-supplemented broth was ca. 635 mosmol/kg, and that for 300 mM $CaCl_2$-supplemented broth was ca. 900 mosmol/kg); the low values are almost certainly due to precipitation of some Ca^{2+}, possibly as phosphate, since a slight but visible precipitate was formed on addition of $CaCl_2$ to the broth.

(300 mM) for 30 min at 37°C showing 8.6% survival under the same challenge conditions.

The NaCl effect is not due to osmotic down-shocking during acid challenge. The acid challenge described above was in pH 3.0 No. 2 broth. This medium has only 85 mM added NaCl and, accordingly, transfer from broth with 300 mM added NaCl to challenge medium could have produced a slight osmotic down-shock, which could have led to acid sensitization (22). NaCl-induced organisms were, however, essentially as acid sensitive when challenged in broth with 300 mM added NaCl, i.e., when not undergoing osmotic down-shock. If an osmotic down-shock was the basis for the sensitization, then preincubation in pH 7.0 aqueous saline (300 mM) should have resulted in enhanced acid killing on challenge, but it did not (Table 2).

Conditions for induction of acid sensitivity. Nalidixic acid, which fully inhibits DNA synthesis at 20 μg/ml, did not prevent acid sensitization (Table 4). In contrast, chloramphenicol (CAP) at 200 μg/ml, which fully and immediately inhibits protein synthesis in strain TA15, allowed appreciable but reduced sensitization when added at the time of NaCl addition but had virtually no effect when added 5 min later (Table 4).

TABLE 4. Effects of inhibitors of macromolecular synthesis on NaCl-induced acid sensitivity[a]

Induction by 300 mM NaCl	Induction time (min)	Inhibitor added during induction (time [min])	% Survival after pH_o 3.0 for 5 min
Expt 1			
No	NA	NA	16.3
Yes	30	None	0.01
Yes	30	CAP (0)	1.0
Yes	30	CAP (5)	0.02
Yes	30	Tetracycline (0)	0.7
Yes	30	Rifampin (0)	0.7
Yes	30	Nalidixic acid (0)	0.04
Expt 2			
No	NA	NA	24.6
Yes	30	None	0.04
Yes	30	CAP (0)	2.0
Yes	30	CAP (−5)	3.1
Yes	60	None	0.01
Yes	60	CAP (0)	3.3
Yes	90	None	0.04
Yes	90	CAP (0)	3.7

[a] Strain TA15 organisms grown to the exponential phase in low-salt broth were transferred to the same medium with the additions shown. In one case, CAP was added 5 min before NaCl. After 30, 60, or 90 min at 37°C, samples were acid challenged (see Materials and Methods). Concentrations: CAP, 200 μg/ml; tetracycline, 5 μg/ml; rifampin, 30 μg/ml; nalidixic acid, 20 μg/ml. Although the acid sensitivity value shown for CAP added at −5 min was slightly greater than that for CAP added at time zero, this only applied to the 5-min challenge period; with acid challenges for 3.5 and 7 min, survival values were 4.2 and 0.6%, respectively, for CAP at time zero and the same (i.e., 4.2 and 0.6%) for CAP at −5 min. NA, not applicable.

When CAP was added with salt at time zero, the acid sensitivity of the CAP-exposed cells (for seven experiments, the mean survival percentage was 1.43% ± 0.36% after 5 min at pH_o 3.0) was significantly greater (99% confidence) than that of uninduced cells (mean survival, 14.4% ± 1.6%) but significantly less (99% confidence) than that of cells induced with NaCl minus CAP (mean survival, 0.08% ± 0.035%). To ensure that all protein synthesis had ceased before induction, CAP was also added at −5 min (i.e., 5 min before NaCl exposure); the effect was similar to that of CAP added at time zero (Table 4 results and footnote). A second protein synthesis inhibitor, tetracycline, also allowed partial sensitization (Table 4), as did the RNA synthesis inhibitor rifampin. Although the effects of CAP, tetracycline, and rifampin suggested that part of the sensitization was protein synthesis dependent, it was possible that full induction could occur with longer periods of CAP exposure; with 60 or 90 min of CAP exposure, however, sensitization was still only partial (Table 4).

Can other monovalent cations induce acid sensitivity? Lithium chloride and ammonium chloride were almost as effective as NaCl, as were NaBr, $NaNO_3$, and Na_2SO_4; potassium chloride and rubidium chloride were, however, significantly less active than NaCl at 300 mM, and cesium chloride had no significant effect (Table 3). Divalent cations were tested and were quite effective.

NaCl-induced acid sensitivity is independent of NhaA and NhaR. Deletion of the major Na^+/H^+ antiporter gene (*nhaA*) did not prevent the NaCl effect in strain NM81, and NaCl led to sensitization in strains OR100 (*nhaR*), OR200 (which has deletions of both *nhaA* and *nhaR*), and RS1 (which has deletions of both *nhaA* and *nhaB*). Additionally, the sodium ion pore inhibitor amiloride had no effect on either induction

of the salt effect (at 1.0 mM) or acid sensitivity of NaCl-induced cells (at 0.3 and 1.0 mM).

Loss of acid sensitivity in low-salt broth. Strain TA15 organisms were grown in broth with 300 mM added NaCl and, after washing to remove the salt, transferred to low-salt broth and incubated at 37°C. Those challenged immediately after induction with NaCl for 30 min at 37°C showed 0.02% survival (pH_o 3.0 for 7 min), and those subsequently incubated in low-salt broth for 90 or 240 min showed 0.05 and 13.5% survival, respectively, whereas uninduced cells gave 10.0% survival with the same challenge exposure.

DISCUSSION

NaCl-induced acid sensitivity undoubtedly involves a phenotypic change in most or all of the exposed organisms. Selection of acid-sensitive mutants would not have been possible because of the rapid induction, and in any case, nalidixic acid at 20 μg/ml, which almost immediately stops division of strain TA15, had no effect on sensitization. This also indicates that induction is independent of DNA synthesis and suggests that it is not dependent on the extent of DNA supercoiling, which sometimes influences induction of stress responses (15). The effects of CAP, tetracycline, and rifampin showed that some sensitization can occur if protein synthesis and RNA synthesis are completely inhibited; full sensitization, however, appeared to depend on the ability of both processes to occur. There was a possibility that CAP, etc., merely slowed down sensitization, i.e., that sensitization was completely protein synthesis independent but not fully mounted in 30 min of exposure to CAP. This possibility was ruled out by the finding that after 60 and 90 min of induction with CAP, sensitization was still only partial (Table 4, experiment 2). NaCl-induced synthesis of the NhaA antiporter or NaCl-associated changes to the properties of the NhaA or NhaB antiporter can be ruled out as the basis for sensitization, as the *nhaA nhaB* deletion strain showed sensitization with NaCl exposure and the acid sensitivity of the NaCl-induced cells was unaffected by amiloride, which inhibits sodium ion pores.

The range of salts which sensitize suggests that it is the rise in osmotic pressure which is the trigger. The efficacy of sucrose (but not glycerol, which freely permeates membranes) as an inducer (Table 3) accords with this, but whereas LiCl, NH_4Cl, and NaCl were almost equally effective inducers, KCl and RbCl were significantly less effective and CsCl did not sensitize, although the osmolalities that the K^+, Rb^+, and Cs^+ salts produced were essentially the same as those produced by the Na^+, Li^+, and NH_4^+ salts (Table 3).

NaCl-induced acid sensitivity is described here. The ASI process (19, 20) also involves two sensitization components; component 1 sensitization is independent of, and component 2 formation is dependent on, protein synthesis. Component 2 sensitization is unrelated to NaCl-induced sensitivity, because induction is inhibited by amiloride and KCl, neither of which affects NaCl induction of acid sensitivity. In contrast, induction of ASI component 1 is, like the NaCl effect, not prevented by amiloride and is not sodium ion specific. Although the induction processes resemble one another, there are three differences which suggest that they are distinct. (i) Whereas ASI component 1 is fully in place after alkalinization for 10 min at 37°C, salt induction requires at least 30 min (Table 2). (ii) Organisms with ASI in place are much less sensitive to acid if amiloride at 1 mM is present during challenge; this agent has no effect on the acid challenge of NaCl-induced cells. (iii) Strain 1157, which shows marked synthesis of both ASI sensitization components (20), is not sensitized by salt, nor is strain MC4100, which shows normal ASI at alkaline pH_o (19).

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