

# Osmotic Stress Leads to Decreased Intracellular pH of *Listeria monocytogenes* as Determined by Fluorescence Ratio-Imaging Microscopy

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**Intracellular pH (pH<sub>i</sub>) of *Listeria monocytogenes* was determined after exposure to NaCl or sorbitol in liquid and solid media (agar). Both compounds decreased pH<sub>i</sub>, and recovery on solid medium was impaired compared to that in liquid medium. *N,N'*-dicyclohexylcarbodiimide abolished pH<sub>i</sub> recovery, and lowering a<sub>w</sub> with glycerol showed no effect on pH<sub>i</sub>.**

*Listeria monocytogenes* is a ubiquitous gram-positive food-borne pathogen, with a widespread occurrence in, e.g., fresh meat and poultry (10), processed ready-to-eat meats (15), seafood (17), soft-style cheeses (18), and butter (19). This frequent occurrence is mainly explained by its ability to survive and grow at conditions associated with normal processing and distribution of food, such as refrigeration temperatures (27), high NaCl concentrations (20), or water activities (a<sub>w</sub>) as low as 0.92 (24). Although *L. monocytogenes* is a neutrophile, it is tolerant to acidic conditions (5, 8), and Shabala et al. (22) demonstrated that *L. monocytogenes* maintained a constant intracellular pH (pH<sub>i</sub>) at extracellular pH (pH<sub>ex</sub>) values as low as 4.0. Information on the effect of osmotic stress on pH<sub>i</sub> of *L. monocytogenes* seems not to be available, and therefore the main objective of the present study was to investigate the relationship between pH<sub>i</sub> of *L. monocytogenes* and osmotic stress in liquid brain heart infusion broth (BHI) as well as on solid BHI (agar) by using fluorescence ratio-imaging microscopy according to the methods of Budde and Jakobsen (4).

The strains of *L. monocytogenes*, EGD (kindly provided by Werner Goebel, Biozentrum, Universität Würzburg, Germany) and 4140 (the Danish Meat Research Institute, Roskilde, Denmark), were grown in BHI adjusted to pH 6.0 with HCl at 37°C for 10 h before use. Fluorescent labeling of the cells and image acquisition were performed essentially as previously described (4, 23), where washed cells were stained with 30 μM 5,6-carboxyfluorescein diacetate-succinimidyl ester at 30°C for 30 min. Excitation with 490- and 435-nm wavelengths was performed through a 510-nm dichroic mirror, and emission was recorded through an 515- to 565-nm band pass filter on a Coolsnap<sub>ex</sub> camera (Photometrics, Tucson, Ariz.). Ratio imagings were performed by using MetaFluor, version 4.5 (Universal Imaging Corporation, West Chester, Pa.), and a minimum of 40 single cells was analyzed in each experiment. For pH calibration, labeled cells were permeabilized with eth-

anol (63% [vol/vol]) and were suspended in either BHI broth or were immobilized on the surface of BHI agar adjusted with HCl or NaOH to a pH in the range 5.5 to 8.5 (steps of 0.5), allowing pH<sub>i</sub> to equilibrate to pH<sub>ex</sub>. A polynomial model fitted to the data was subsequently used to calculate pH<sub>i</sub>.

In liquid BHI the labeled cells were immobilized on the surface of an 8-well chamber slide (Lab-Tek II Chambered cover glass; Nalge Nunc International) by adding 100 μl of bacterial suspension and allowing the cells to settle, followed by a brief rinse with phosphate-buffered saline before addition of liquid BHI (200 μl) adjusted to the desired pH and containing the desired concentrations of NaCl, sorbitol, or glycerol. For investigations on solid BHI, 15 g of agar/liter was added to the liquid BHI, and 1 ml of molten BHI agar (pH 6.0) containing various levels of NaCl, sorbitol, or glycerol was placed in the concavity of a special microscope slide (Micro Culture One Well; VWR International, Albertslund, Denmark). A coverslip was placed on top of the molten agar to provide a flat surface, and after solidification the coverslip was removed, 10 μl of labeled cells was spread onto the agar surface, and a fresh coverslip was placed on the agar when the liquid was absorbed. This absorption introduced a time lag, prohibiting the acquisition of images at time zero in the solid BHI system. However, the pH<sub>i</sub> value of the control experiment was essentially constant and was chosen to represent the initial pH<sub>i</sub> of the cells in the agar experiments.

In contrast to earlier findings dealing with osmotically stressed *Escherichia coli* (9), we did not observe any transient increase in pH<sub>i</sub> of *L. monocytogenes* during the initial phase of exposure to osmotic stress. On the contrary, we observed a significant decrease in pH<sub>i</sub> following exposure to high concentrations of NaCl (Fig. 1 and 2). For clarity, error bars have been excluded from the figures, but the standard error mean of the pH<sub>i</sub> measurements in all figures were typically in the range of 0.01 and 0.05 and never exceeded 0.1. This very homogenous response indicates homogenous cultures, which would also be expected from the growth conditions. However, it is important to note that one of the greatest benefits of this method is the ability to evaluate the heterogeneity of a culture due to single-cell analysis, as previously demonstrated (4).

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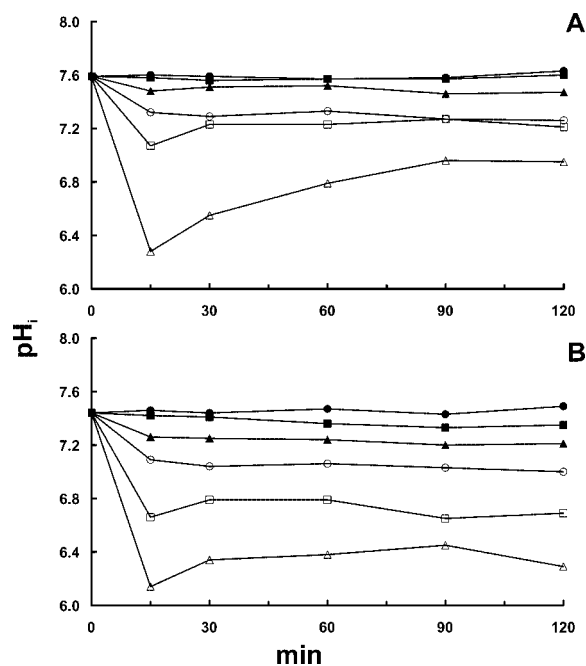


FIG. 1. Effect of adding NaCl to liquid BHI (A) and solid BHI (B) on  $pH_i$  of *L. monocytogenes* 4140 at a  $pH_{ex}$  of 6.0. Fluorescent images were acquired at 15-min intervals, and each point represents the average  $pH_i$  of 40 randomly selected cells. Symbols indicate NaCl concentrations of 0 M (full circle), 0.5 M (full square), 1.0 M (full triangle), 1.5 M (open circle), 2.0 M (open square), and 2.5 M (open triangle).

Comparing liquid BHI (Fig. 1A and 2A) and solid BHI (Fig. 1B and 2B), it is seen that the effect of NaCl on  $pH_i$  was more pronounced on the agar substrate, especially regarding the ability to restore  $pH_i$ . Figure 1 shows that after 2 h of incubation,  $pH_i$  reaches 6.8 and 6.3 on agar with 2.0 and 2.5 M NaCl; pH values when cells were exposed in broth were 7.2 and 6.8. For *L. monocytogenes* EGD, similar effects of NaCl concentrations were observed on  $pH_i$  but with the noteworthy exception that this strain was not able to restore the gradient at all after 1.5 h of incubation on agar containing 2.5 M NaCl (Fig. 2B). When growth was investigated by using optical density measurements, addition of 2.0 M NaCl completely inhibited growth of strain 4140 for 24 h, whereas 1.5 M NaCl was sufficient to inhibit strain EGD (unpublished results).

To examine whether this  $pH_i$  regulation was specific for the ionic compound NaCl, *L. monocytogenes* was also exposed to osmotic stress by using the nonionic compound sorbitol in the same molar concentrations as that used for NaCl. The results showed almost the same effect of sorbitol as NaCl, although the initial decrease in  $pH_i$  was less pronounced with sorbitol in liquid BHI, as seen for *L. monocytogenes* 4140 in Fig. 3A. The recovery on agar was impaired (Fig. 3B) compared to that in liquid (Fig. 3A), but the strain variety observed with NaCl was not observed with sorbitol, as the recovery of *L. monocytogenes* EGD was almost identical to that of strain 4140 (data not shown).

All water activities were determined with an Aqualab CX-2 (Decagon Devices, Inc., Pullman, Wash.) at 25°C, and the  $a_w$  values observed in the sorbitol samples were higher than those

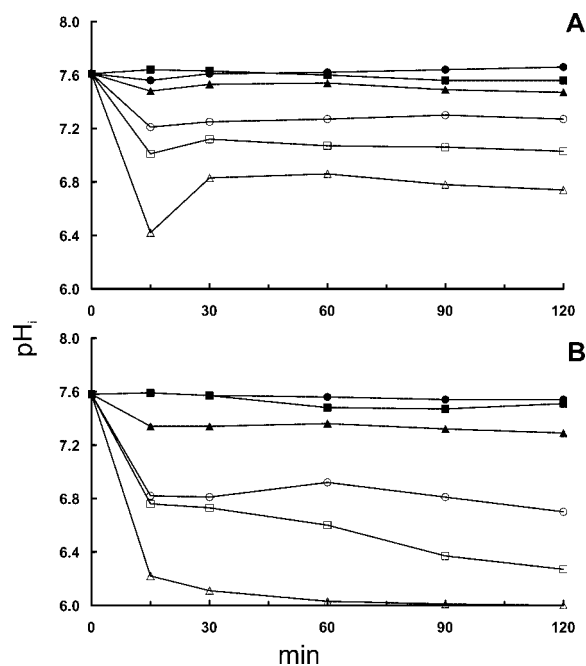


FIG. 2. Effect of adding NaCl to liquid BHI (A) and solid BHI (B) on  $pH_i$  of *L. monocytogenes* EGD at a  $pH_{ex}$  of 6.0. Fluorescent images were acquired at 15-min intervals, and each point represents the average  $pH_i$  of 40 randomly selected cells. Symbols indicate NaCl concentrations of 0 M (full circle), 0.5 M (full square), 1.0 M (full triangle), 1.5 M (open circle), 2.0 M (open square), and 2.5 M (open triangle).

in NaCl at identical molar concentrations (Table 1). When glycerol was added to liquid BHI until  $a_w$  was 0.94,  $pH_i$  remained constant around 7.6 (results not shown), similar to the control experiments with an  $a_w$  of 0.995 (Table 1). This finding is not surprising, as glycerol is membrane permeant and therefore does not convey an osmotic stress. However, it underlines that  $a_w$  per se does not affect  $pH_i$  regulation, and therefore the observed  $pH_i$  decrease in the NaCl and sorbitol samples cannot simply be attributed to a decreased  $a_w$ , which suggests that other physicochemical properties of the  $a_w$ -controlling solutes are involved (14).

The  $pH_i$  recovery of *L. monocytogenes* following a sharp immediate decrease may be explained by osmoadaptation of the organism, most probably caused by accumulation of compatible solutes like glycine betaine and carnitine (1, 12, 25), increased proton pumping by the  $H^+$ -ATPase (6), and consumption of intracellular protons by glutamate decarboxylase (7). Failure of bacteria to recover homeostasis under severe osmotic stress might reflect ATP depletion, which will inhibit the ATP-dependent uptake of glycine betaine and carnitine (11, 13, 29), or a decrease in the  $H^+$ -ATPase activity (6).

To determine if  $pH_i$  recovery was  $H^+$ -ATPase dependent, cells were exposed to a 0.1 mM concentration of the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD) in liquid BHI and 0.1 mM DCCD in combination with 2.5 M NaCl. Table 2 shows that treatment of *L. monocytogenes* with DCCD only decreased  $pH_i$  a little, from 7.6 to 7.3 during 60 min of exposure, whereas exposure to 2.5 M NaCl shows the decrease and slow recovery also observed in Fig. 1A. However, exposure to

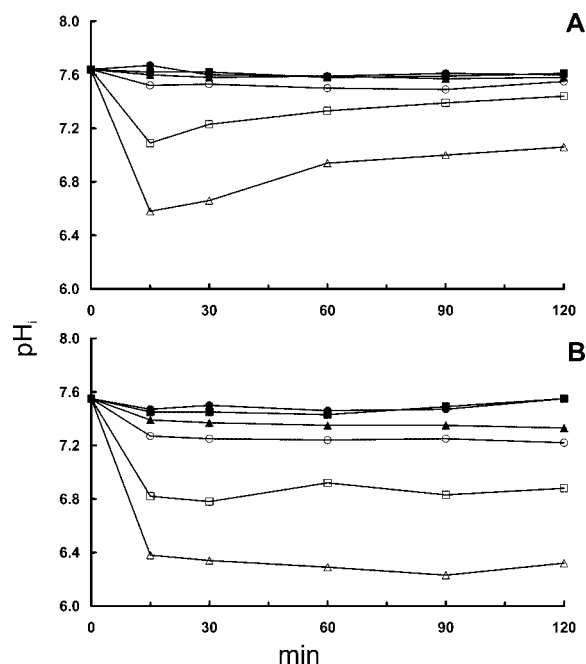


FIG. 3. Effect of adding sorbitol to liquid BHI (A) and solid BHI (B) on  $\text{pH}_i$  of *L. monocytogenes* 4140 at a  $\text{pH}_{\text{ex}}$  of 6.0. Fluorescent images were acquired at 15-min intervals, and each point represents the average  $\text{pH}_i$  of 40 randomly selected cells. Symbols indicate NaCl concentrations of 0 M (full circle), 0.5 M (full square), 1.0 M (full triangle), 1.5 M (open circle), 2.0 M (open square), and 2.5 M (open triangle).

2.5 M NaCl for 15 min followed by exposure to 2.5 M NaCl and 0.1 mM DCCD was more detrimental to the bacteria: the decrease in  $\text{pH}_i$  was pronounced, and there was no apparent  $\text{pH}_i$  recovery after 60 min, which strongly indicates that ATPase has a role in the recovery of  $\text{pH}_i$  after exposure to osmotic stress. A previous study has shown similar synergistic effect between a compound affecting the proton permeability (ethanol) and osmotic stress imposed by NaCl or sucrose (2).

At a  $\text{pH}_{\text{ex}}$  value of 5.0 the cells in the control experiment were able to maintain homeostasis, as the  $\text{pH}_i$  was constant within the interval 6.8 to 7.0 (Fig. 4). Addition of 2.0 and 2.5 M NaCl at  $\text{pH}_{\text{ex}}$  5.0 caused a pronounced  $\text{pH}_i$  reduction for *L. monocytogenes* 4140 in liquid BHI (Fig. 4), and the decrease after exposure to 2.0 M NaCl was particularly more pronounced than the effects observed at  $\text{pH}_{\text{ex}}$  6.0 (Fig. 1A). The

TABLE 1.  $a_w$  of BHI with different additions of NaCl and sorbitol at pH 6.0

Additive concn (M)	$a_w$			
	Liquid BHI		Solid BHI	
	NaCl	Sorbitol	NaCl	Sorbitol
0	0.995	0.995	0.997	0.997
0.5	0.987	0.991	0.975	0.987
1.0	0.969	0.980	0.961	0.976
1.5	0.954	0.967	0.940	0.966
2.0	0.934	0.955	0.920	0.951
2.5	0.908	0.940	0.906	0.929

TABLE 2. Regulation of  $\text{pH}_i$  in *L. monocytogenes* 4140 after addition of DCCD and NaCl to liquid BHI at pH 6.0

Time (min)	$\text{pH}_i$ of 40 randomly selected cells (average $\pm$ SEM) in:			
	BHI (control)	BHI + 0.1 mM DCCD	BHI + 2.5M NaCl	BHI + NaCl + DCCD <sup>a</sup>
0	7.62 $\pm$ 0.01	7.61 $\pm$ 0.01	7.61 $\pm$ 0.01	7.62 $\pm$ 0.01
30	7.62 $\pm$ 0.01	7.30 $\pm$ 0.04	6.84 $\pm$ 0.03	5.61 $\pm$ 0.01
60	7.66 $\pm$ 0.01	7.30 $\pm$ 0.06	6.99 $\pm$ 0.02	5.64 $\pm$ 0.01

<sup>a</sup> Cells were exposed to 2.5 M NaCl for 15 min, and the medium was then replaced by liquid BHI containing 2.5 M NaCl and 0.1 mM DCCD.

recovery of  $\text{pH}_i$  after exposure to high concentrations of NaCl at  $\text{pH}_{\text{ex}}$  5.0 was marginal within the 2 h of incubation (Fig. 4), which suggests a synergistic effect of low  $\text{pH}_{\text{ex}}$  and NaCl on the  $\text{pH}_i$  regulation in *L. monocytogenes*.

The observed discrepancies between the  $\text{pH}_i$  regulations of bacteria on a structured matrix and those of a liquid environment of similar nutritional composition have not been reported previously, and the results are consistent with those of previous studies that revealed a decreased growth rate in solid medium compared to that in liquid medium (3). The most likely explanation for the discrepancy in the behavior of bacteria in liquid and on solid media is decreased diffusion rates in the solid matrix (28), as the chemical composition of the liquid and solid BHI are identical, with the exception of agar added to the solid system. Strain EGD was more impaired in  $\text{pH}_i$  recovery on agar containing high concentrations of NaCl (1.5 to 2.5 M) (Fig. 2B) than was strain 4140. Among strains of *L. monocytogenes* investigated, strain EGD was very sensitive to gastric fluid (pH 2.5), which was linked to a low glutamate decarboxylase activity (7). The presence of NaCl and sorbitol in our solid system caused a pronounced decrease in  $\text{pH}_i$ , and it is possible that the ensuing lack of recovery of strain EGD in the agar containing NaCl is due to interactions between NaCl and mechanisms regulating  $\text{pH}_i$ , such as the glutamate decarboxylase.

During image analysis, it was noticed that the individual cells were shrinking considerably when NaCl or sorbitol was added in high concentrations, which suggests a loss of water. This is generally accepted as a response to osmotic upshifts (29) and

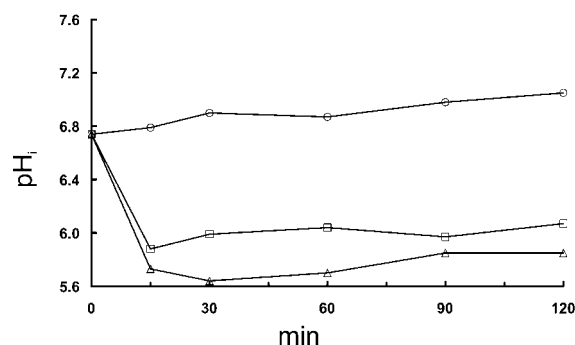


FIG. 4. Effect of adding increasing levels of NaCl to liquid BHI on  $\text{pH}_i$  of *L. monocytogenes* 4140 at a  $\text{pH}_{\text{ex}}$  of 5.0. Addition of 0 M NaCl is indicated with circles, 2.0 M NaCl is indicated with squares, and 2.5 M NaCl is indicated with triangles. Each data point represents the average  $\text{pH}_i$  of 40 randomly selected bacteria cells.

is consistent with our  $pH_i$  measurements, as a water loss would increase proton concentration and hence decrease  $pH_i$ . A direct correlation between immediate cell shrinkage caused by osmotic stress and immediate  $pH_i$  decrease was recently described for yeasts (26), but to our knowledge this correlation has not been demonstrated for bacteria. The present study demonstrates that the initial intracellular response to osmotic stress in *L. monocytogenes* is a decrease in  $pH_i$ , which is also a reported response to acid stress (16, 22), and therefore it provides a direct physiological explanation for the cross-protection against osmotic stress that is observed after induction of acid tolerance response in *L. monocytogenes* (21).

In conclusion, the present study demonstrates that the initial response of an osmotic shock caused by NaCl or sorbitol is a drop in  $pH_i$ . The rapid drop in  $pH_i$  is followed by a slower recovery, where the magnitude of recovery is dependent on both solute concentration and solute type. Finally, the study demonstrates that the  $pH_i$  recovery is reduced on a solid medium compared to that in a liquid medium. This underlines the recently raised issue that caution needs to be used when results obtained in liquid model systems are extrapolated to predict the fate of bacteria in structured foods (3).

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