APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 4123–4126 Vol. 63, No. 10
0099-2240/97/$04.00+0

Factors Controlling Acid Tolerance of *Listeria monocytogenes*: Effects of Nisin and Other Ionophores

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Received 31 March 1997/Accepted 11 August 1997

The acid tolerance of a *Listeria monocytogenes* serotype 4b strain was studied by measuring its ability to survive at an acidic pH at 37°C. The acid tolerance of *L. monocytogenes* was much lower than those of *Escherichia coli* O157:H7 and *Shigella flexneri* strains. This observation suggested a higher infective dose for *L. monocytogenes* than *E. coli* O157:H7 and *Shigella*. The susceptibility of *L. monocytogenes* to acidic pH was dependent upon growth medium pH and growth phase of the culture. Nisin and some other ionophores reduced the acid tolerance of both stationary-phase and log-phase cultures of *L. monocytogenes*. These studies indicated that nisin might be a useful candidate for controlling acid tolerance of *L. monocytogenes*.

Bacterial pathogens can often survive under harsh environmental conditions, including extremes of pH, high osmolarity, and extremes of temperature. In particular, the low pH plays a very important role in survival of food-borne pathogens. In order to cause illness, an invading organism must survive the acidic environment of the stomach before it reaches the intestine. Thus, the acidity of gastric juice provides a first line of defense against food-borne pathogens. Reduction in gastric acidity has been associated with an increase in survival rate of some common food-borne pathogens in humans (25) and a lower infective dose in animal models (9, 27). Gorden and Small (14) demonstrated that *Shigella* spp. are more acid resistant (pH 2 to 2.5) than are *Salmonella* spp. and *Escherichia coli* and hypothesized that the high acid resistance of *Shigella* spp. is a major contributing factor to their relatively low (10 to 500 organisms) infective dose. *E. coli* O157:H7 strains were also found to be highly resistant to low pH (6, 21, 31), which is in accordance with epidemiological studies indicating low infective doses (5, 15). Acid tolerance also plays an important role in survival of intracellular pathogens, e.g., *Listeria* and *Shigella*. Not only does the infecting organism have to survive the low intracellular pH of the phagolysosome, but also the appropriate virulence genes must be expressed at that pH. Additionally, in the case of food-borne pathogens the ability to survive and multiply in acidic foods (apple cider, mayonnaise, and sausage, etc.) and in food artificially treated with organic acids for preservation also plays an important role in determining the infective dose.

Listeria monocytogenes, a ubiquitous, gram-positive, intracellular pathogen, is the causative agent of human listeriosis. The disease is characterized by septicemia, meningitis, and abortions, often causing 30 to 40% mortality among neonates and immunocompromised individuals (12). Epidemiological studies have indicated that both epidemic and sporadic cases of human listeriosis are food-borne (28). *L. monocytogenes* has been isolated from virtually every kind of food, including acidic foods (e.g., sausage and cheese). In this report, we describe experiments performed to clarify the mechanism and the factors which modify acid tolerance in *L. monocytogenes*. We also studied the effects of several ionophores, including nisin, on the acid tolerance of *L. monocytogenes*. An increased understanding of acid tolerance should be useful in controlling this organism in food and should also be useful in predicting the infective dose of *L. monocytogenes*.

Acid tolerance of *L. monocytogenes*. The acid tolerance of *L. monocytogenes* was studied by comparing the viability of LS2 with that of two well-known highly acid-tolerant food-borne pathogens, *E. coli* O157:H7 ATCC 43895 and a *Shigella flexneri* strain (6). LS2, an *L. monocytogenes* serotype 4b strain, was isolated from a patient during a milk-related outbreak in Massachusetts (12). Bacterial strains were maintained at −70°C in the presence of 25% glycerol. The test strains were routinely grown in Trypticase soy broth containing 0.6% yeast extract (TSBYE) in 125-ml flasks at 37°C in a water bath shaker for about 16 h (overnight). The total numbers of CFU in the overnight cultures were 2×10^9 to 5×10^9/ml, which corresponds to an A_{600} of 1.6 to 1.8. The cultures were centrifuged, washed once, and suspended in the same volume of TSBYE. They were immediately diluted 1:100 in 5 to 10 ml of TSBYE adjusted to pH 3.0 with HCl and incubated at 37°C with gentle shaking. Samples were periodically withdrawn, diluted appropriately with saline (0.85% NaCl in distilled water), and plated onto Trypticase soy agar containing 0.6% yeast extract (TSAYE). Colonies were counted after 24 to 48 h of incubation at 37°C. Each assay point represents an average number of CFU from at least two plates in any given experiment. The viability of LS2 was reduced by a factor of 4 log units within 180 min at 37°C, while for the *Shigella* strain the reduction was only 30-fold and for the *E. coli* O157:H7 strain no reduction of viability was observed at pH 3.0 (Fig. 1). We also determined the survival of *L. monocytogenes* (LS2 and LS111), *S. flexneri*, and *E. coli* O157:H7 in TSBYE adjusted to pH 2.0, 3.0, 4.0, 5.0, and 6.0. Results (data not shown) indicated that *Listeria* strains LS2 and LS111 are much more sensitive at a pH ranging from 2.0 to 5.0 than the *Shigella* and *E. coli* O157:H7 strains. These experiments confirmed our observation (Fig. 1) that *L. monocytogenes* is, in general, less acid tolerant than *Shigella* and *E. coli* O157:H7 and suggest that *L. monocytogenes* has a much higher infective dose than *E. coli* O157:H7 and *Shigella*. Results from the epidemiological studies of sporadic and epidemic listeriosis cases (12, 28) are consistent with this assumption. The higher sensitivity of *Listeria* strains (data not shown) even at pH 4.0 and 5.0 also suggested that the low-pH food

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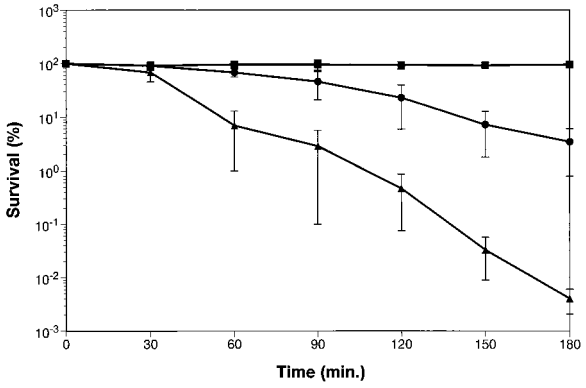

FIG. 1. Survival of *L. monocytogenes* LS2 (▲), *S. flexneri* (●), and *E. coli* O157:H7 (■) in TSBYE, pH 3.0. Data are averages for three independent experiments. Error bars show standard deviations.

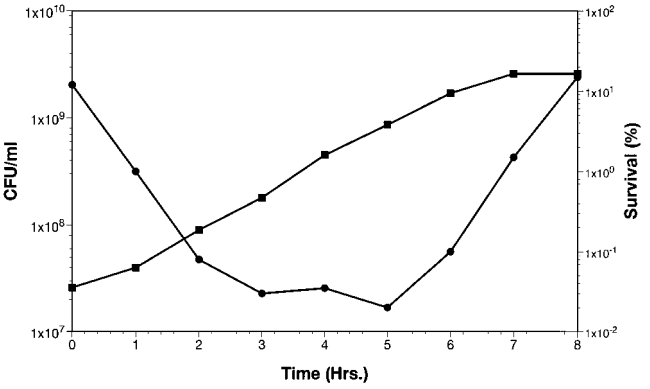

FIG. 2. Growth phase-dependent acid tolerance of *L. monocytogenes* LS2 grown in TSBYE, pH 7.5 (■). Survival percentages (●) were calculated after 60 min of exposure in TSBYE, pH 3.0. Data are representative of three independent experiments.

preservation might be more effective for *L. monocytogenes* than for *Shigella* and *E. coli* O157:H7 strains.

Growth medium pH and acid tolerance. Bacterial resistance to low pH can be substantially increased by exposing the cells to a sublethal pH before the lethal pH challenge (3, 13, 29). The sublethal acid induction also provides cross protection against other stress factors (13, 18, 19, 24). The effect of sublethal-pH-mediated induction of acid tolerance (adaptation) in *L. monocytogenes* was investigated by survival studies with the cultures grown to stationary phase in TSBYE buffered at different pHs. The results from a typical experiment are given in Table 1. Survival was inversely correlated with the growth medium pH. A susceptibility increase of about 4 log units was observed when cells were grown in pH 8.0 compared with the cells grown in pH 5.5. Our results agreed well with those of previous works (17, 24), which showed that a brief period (1 to 2.5 h) of adaptation to sublethal pH 5.0 could render log-phase cells of *L. monocytogenes* more resistant to subsequent challenge to low pH (pH 3.0 to 3.5). Although growth in organic acids failed to induce the acid tolerance response in *Salmonella typhimurium* (3), our results with citric acid (pH 5.5) indicate (Table 1) that induction of acid tolerance by organic acids is possible in *L. monocytogenes*. As many foods either are naturally acidic or are modified by the addition of organic acids for preservation, the surviving bacteria might be more resistant to subsequent low-pH challenge and other lethal treatments.

Role of growth phase in acid tolerance. Most bacterial pathogens exhibit a higher level of resistance against environmental stresses, including acid stress at stationary phase (2, 6, 10, 14, 16). A key factor in the stationary-phase-induced stress response is induction of the *rpoS* regulon that is brought about by the nutrient-limiting condition of the stationary-phase cultures (16, 20). Such nutrient-limiting conditions are also present in different foods and other environments (26). The effect of growth phase on acid tolerance was studied by growing LS2 in TSBYE containing 100 mM Trizma (for pH 7.5) or 100 mM citrate (for pH 5.5). These overnight cultures were diluted 1:100 in fresh TSBYE (pH 7.3 or 5.5) and incubated at 37°C with gentle shaking in 125-ml flasks. Samples (0.5 ml) were periodically withdrawn in 1.5-ml Eppendorf tubes and centrifuged at 15,000 × *g* for 3 min. The pellets were suspended in same volume of TSBYE adjusted to pH 3.0 with HCl. These cultures were plated immediately (0 min) and after 60 min of incubation at 37°C for viable counts. Figures 2 and 3 show results from the experiments with LS2 cells grown at different pHs. The acid tolerance of the overnight culture (late stationary phase) was about 500-fold higher than that of the mid-log-phase culture. As the culture grew beyond mid-log phase, the acid tolerance increased and reached a maximum, again, at late stationary phase. Similar observations were also made with *E. coli* O157:H7 (6) and *Shigella* (14), which indicated that the food-borne pathogens probably possess a common mechanism to achieve high levels of acid resistance at stationary phase.

TABLE 1. Survival in TSBYE (pH 3.0) of stationary-phase cultures of *L. monocytogenes* LS2 preconditioned at various pHs

Preconditioning pH[a]	Survival (%)[b]
5.5	12
6.0	1.0
6.5	2.0
7.0	1.0×10^{-2}
7.5	1.0×10^{-2}
8.0	3.0×10^{-3}

[a] TSBYE was buffered with 100 mM citrate (for pH 5.5), 2-(*N*-morpholino) ethanesulfonic acid (for pH 6.0 and 6.5), 3-(*N*-morpholino)propanesulfonic acid (for pH 7.0), and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, or Trizma (for pH 7.5 and 8.0).

[b] Calculated from viable counts of the cultures following 60 min of incubation at 37°C in TSBYE acidified to pH 3.0 with HCl.

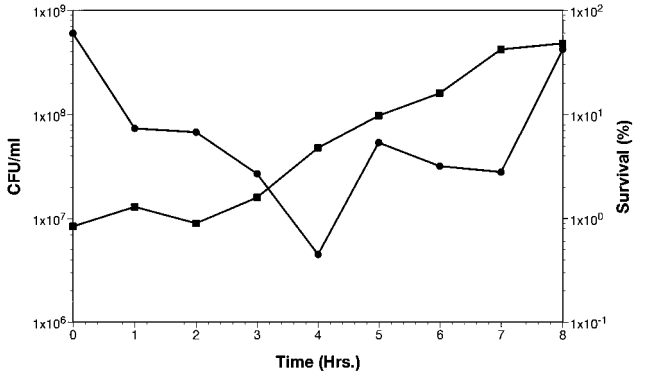

FIG. 3. Growth phase-dependent acid tolerance of *L. monocytogenes* LS2 grown in TSBYE, pH 5.5 (■). Survival percentages (●) were calculated after 60 min of incubation at 37°C in TSBYE, pH 3.0. Data are representative of three independent experiments.

TABLE 2. Survival of *L. monocytogenes* LS2 in the presence of various ionophores

Substance[a]	Concn	Survival (%)[b] of culture	
		Stationary phase	Log phase
Alcohol	1%	100	6.2×10^{-2}
Gramicidin	10 μg/ml	2.8×10^{-4}	$<10^{-6}$
Nigericin	5 μg/ml	7.1×10^{-4}	$<10^{-6}$
CCCP	10 μg/ml	2.5	$<10^{-6}$
DCCD	1 mM	1.0	9.4×10^{-4}

[a] 100× stock solutions of gramicidin, nigericin, CCCP, and DCCD were prepared in absolute alcohol, filter sterilized with 0.2-μm-pore-size syringe filters, and diluted 1:100 before use.
[b] Calculated following 60 min of incubation at 37°C in TSBYE adjusted to pH 3.0 with HCl.

Our results also demonstrate that the growth medium pH-induced acid tolerance appears to be independent of the growth phase. A comparison of Fig. 2 and 3 reveals that the cells grown at pH 5.5 exhibited more resistance (two- to fivefold more) at all phases of growth than the cells grown at pH 7.5 (Fig. 2). These results suggest that the effects of growth phase and low pH adaptation on acid tolerance are to some extent additive and therefore probably work via different, albeit overlapping, mechanisms. Similar differences in acid tolerance between acid-adapted stationary-phase cultures and nonadapted stationary-phase cultures have also been reported for *Salmonella* (13).

Effects of ionophores on acid tolerance. *L. monocytogenes*, like most of the food-borne bacterial pathogens, is neutrophilic. Neutrophiles maintain their internal pH between 6 and 8 in spite of the large variation in the external pH. When the difference between internal pH and external pH is high, the pH homeostasis collapses and the organism dies. The mechanism by which *L. monocytogenes* and other neutrophiles achieve pH homeostasis is not clearly understood (4, 7). Several possibilities have been proposed, including (i) an increase in cytoplasmic buffering capacity, (ii) low proton permeability of the cell membrane, and (iii) the extrusion of protons by a membrane-bound proton pump.

To investigate the mechanism by which *L. monocytogenes* achieves acid tolerance, we studied the effect of four ionophores on survival of LS2 at pH 3.0 at 37°C. Ionophores are a group of compounds that increases the ionic permeability of the cell membrane (30). It is clear from Table 2 that survival in the presence of ionophores was dependent upon the growth phase of the cultures. Gramicidin and nigericin had strong effects on both stationary-phase and log-phase cultures, whereas carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and dicyclohexylcarbodiimide (DCCD) had marginal effects on stationary-phase cultures but very strong effects on log-phase cultures. None of the compounds affected the survival of either stationary-phase or log-phase cultures at pH 7.3 under otherwise identical experimental conditions, indicating that the effect is specific to low-pH-induced lethality and not due to any general toxic reaction. Although these four compounds dissipate proton motive force (PMF), they do so by different mechanisms (23). Gramicidin is an ionophore which renders the cytoplasmic membrane permeable to K^+, Na^+, H^+, and other cations. Nigericin is a K^+/H^+ antiporter and thus dissipates pH gradients in an electroneutral manner. DCCD is a potent inhibitor of membrane-bound proton-translocating ATPase, which is involved in either ATP generation or hydrolysis of ATP to generate PMF and extrude protons from the cytoplasm. CCCP is a respiratory uncoupler which acts as a protonophore, creating H^+ equilibrium between cytoplasmic pH and outside pH. From Table 2, it is apparent that in stationary-phase cells, resistance to low pH is mainly achieved by impermeability to protons, which is readily destroyed by gramicidin and nigericin, thereby increasing the susceptibility by 5 to 6 log units. The marginal effect of CCCP and DCCD is probably due to low rates of metabolism in the stationary-phase cells. On the other hand, in the actively metabolizing log-phase cultures, CCCP and DCCD had as much effect as gramicidin and nigericin, suggesting the active role of ATPase and respiratory coupling in acid tolerance. Thus, our results suggest that the stationary-phase cells use a different mechanism than the log-phase cells to achieve acid tolerance.

Effect of nisin on acid tolerance. Conventional food preservation is mainly achieved by addition of organic acids (low pH), chemicals which increase the osmolarity (low water activity), and low temperature. In recent years, bacteriocins have been increasingly investigated for their ability to preserve food against bacteria (1). Bacteriocins are low-molecular-weight proteins produced by lactic acid bacteria and are very effective for controlling growth of gram-positive organisms. Of all the known bacteriocins, nisin is the only one approved as "generally recognized as safe" by the Food and Drug Administration for use in some cheese products. The antibacterial properties of nisin and other bacteriocins have been associated with their ability to form pores in the membrane, resulting in complete dissipation of PMF (8, 23).

Our studies with gramicidin and nigericin led us to examine the effect of nisin on the acid tolerance of *L. monocytogenes*. The results (Table 3) showed that a small amount of nisin made LS2 cells much more acid sensitive. The effect was more pronounced in log-phase cultures (2×10^8 to 4×10^8 CFU/ml) than in stationary-phase cultures (2×10^9 to 5×10^9 CFU/ml). It was also dependent on the concentration of nisin. Although nisin is bacteriocidal at high concentrations (about 2×10^3 U/ml) even at neutral pH (22), the concentrations used in our experiments were ineffective in reducing viability at pH 7.3. This indicates that the effect (Table 3) was specific to low-pH-mediated viability. Results from these studies suggest that nisin may increase the infective dose of *L. monocytogenes* by increasing sensitivity to the low pH of gastric juice. Nisin was also effective in reducing the viability of LS2 in low-pH medium acidified with citric acid (11), suggesting that nisin may also reduce the growth of *L. monocytogenes* in acidic foods.

TABLE 3. Survival of *L. monocytogenes* LS2 in the presence of nisin

Nisin concn (μg/ml)[a]	Survival (%)[b] in culture	
	Stationary phase	Log phase
0	100	4.6×10^{-2}
25	47	1.9×10^{-3}
125	1×10^{-1}	5.9×10^{-4}
250	7.0×10^{-2}	$<10^{-5}$

[a] Nisin in milk protein (N-5764; Sigma Chemical Co.) was suspended in 0.02 N HCl in saline at a concentration of 10 mg/ml. This preparation contained 2.5% pure nisin, and the final concentrations of nisin were calculated on this basis. According to the manufacturer, each microgram of pure nisin roughly corresponds to 1 U of nisin. The suspension was centrifuged, and the supernatant was filter sterilized with 0.2-μm-pore-size syringe filters and used as a stock solution (250 μg/ml) of nisin. For lower concentrations, the stock solution was diluted in 0.02 N HCl in saline.
[b] Calculated following 30 min of incubation at 37°C in 0.02 N HCl in saline or in nisin solutions adjusted to pH 3.0.

We thank Tom Cebula for his support and critical reading of the manuscript.

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