

## Acid Adaptation of *Listeria monocytogenes* Can Enhance Survival in Acidic Foods and during Milk Fermentation

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We have previously shown that tolerance to severe acid stress (pH 3.5) can be induced in *Listeria monocytogenes* following a 1-h adaptation to mild acid (pH 5.5), a phenomenon termed the acid tolerance response (ATR) (B. O'Driscoll, C. G. M. Gahan, and C. Hill, *Appl. Environ. Microbiol.* 62:1693–1698, 1996). In an attempt to determine the industrial significance of the ATR, we have examined the survival of adapted and nonadapted cells in a variety of acidic foods. Acid adaptation enhanced the survival of *L. monocytogenes* in acidified dairy products, including cottage cheese, yogurt, and whole-fat cheddar cheese. Acid-adapted *L. monocytogenes* cultures also demonstrated increased survival during active milk fermentation by a lactic acid culture. Similarly, acid-adapted cells showed greatly improved survival in low-pH foods (orange juice and salad dressing) containing acids other than lactic acid. However, in foods with a marginally higher pH, such as mozzarella cheese, a commercial cottage cheese, or low-fat cheddar cheese, acid adaptation did not appear to enhance survival. We have previously isolated mutants of *L. monocytogenes* that are constitutively acid tolerant in the absence of an induction step (O'Driscoll et al., *Appl. Environ. Microbiol.* 62:1693–1698, 1996). In the present study, one such mutant, ATM56, demonstrated an increased ability to survive in low-pH foods and during milk fermentation when compared with the wild-type strain. Significant numbers of ATM56 could be recovered even after 70 days in both whole-fat and low-fat cheddar cheese. Collectively, the data suggest that ATR mechanisms, whether constitutive or induced, can greatly influence the survival of *L. monocytogenes* in low-pH food environments.

*Listeria monocytogenes* has been implicated as the causative agent of both epidemic and sporadic food-borne illness. Well-documented outbreaks of listeriosis have been associated with consumption of a number of foods including pasteurized milk, raw milk, cheese, coleslaw, and paté (3, 8). These incidents have heightened the awareness of *L. monocytogenes* as a public-health problem and have increased efforts to prevent further outbreaks. The association of listeriosis with consumption of processed foods has prompted a number of studies to determine the impact of food processing and preservation procedures on the survival of *L. monocytogenes* (reviewed in reference 3). In contrast, the ability of the pathogen to adapt to stress conditions associated with food processing has received little attention. It is evident that *L. monocytogenes* responds to the presence of environmental stressors such as sodium chloride, heat, nitrite, and sorbate, by regulating the synthesis of a number of proteins, including specific virulence factors (22, 23, 25, 26). However, the molecular mechanisms used by *L. monocytogenes* to survive environmental or process-induced stresses have not yet been established.

The ability to tolerate acid stress provides a useful model for analyzing the physiology of adaptive tolerance responses. By using nutrient broth systems, *L. monocytogenes* has been shown to exhibit a significant adaptive acid tolerance response (ATR) following exposure to mild acid (pH 5.5), which is capable of protecting cells from normally lethal acid stress (pH 3.5) (13, 18, 24). We have recently demonstrated that adaptation of *Listeria* cultures to mild acid conditions for 1 h also provides cross-protection against other stresses including osmotic stress and heat shock (24). Adaptation of *L. monocytogenes* to sub-

optimal growth environments therefore confers the ability to alter cellular physiology such that the pathogen becomes resistant to further stress. It is also noteworthy that repeated exposure to low pH can select for constitutively acid-tolerant mutants, one of which has been shown by us to exhibit increased virulence relative to the parental strain (24).

The ATR has been studied most extensively in *Salmonella typhimurium* and *Escherichia coli*. In these organisms, the altered synthesis of a number of proteins, including certain outer membrane and heat shock proteins, is thought to provide a mechanism for maintaining intracellular homeostasis in normally lethal environments (5–7, 12). Recent studies have demonstrated that the ATR has the potential to influence the survival of both *E. coli* and *Salmonella* spp. in foods of low pH. Acid-adapted *Salmonella* spp. and *E. coli* demonstrate significantly increased survival in foods when compared with nonadapted organisms (19, 20). Because a number of foods rely on low pH to prevent the growth of pathogens and spoilage organisms, the ability of food-borne pathogens to initiate an ATR may be an important factor influencing their survival in normally lethal food environments. This study was therefore undertaken to examine the influence of acid adaptation on the survival of *L. monocytogenes* in foods of low pH.

### MATERIALS AND METHODS

**Bacterial strains and media.** *L. monocytogenes* LO28 (serotype 1/2c) is a clinical isolate obtained from P. Cossart, Institut Pasteur, Paris, France. The culture medium used for bacterial growth was tryptone soya broth (Sigma Chemical Co., St. Louis, Mo.) supplemented with 0.6% yeast extract (TSB-YE). Cell growth in broth was monitored as turbidity at 600 nm. *Listeria* cells were isolated from foods by direct plating onto *Listeria* selective agar (LSA) (Oxford formula; Oxoid, Columbia, Md.).

A lyophilized starter culture, *Streptococcus thermophilus* (Chr. Hansens, Little Island, Cork), used in milk fermentation experiments was routinely propagated at 37°C and stored at 4°C. Deep-frozen lactic cultures L253 (*Lactococcus cremoris*, *Lactococcus lactis*, and *Leuconostoc cremoris*) (Chr. Hansens) used in the preparation of cottage cheese were stored at –65°C. Fresh unripened mozzarella

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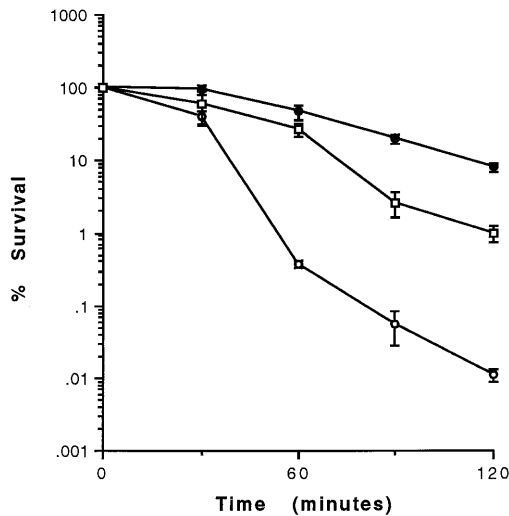


FIG. 1. ATR in *L. monocytogenes*. The survival of acid-adapted (●) and nonadapted (○) *L. monocytogenes* LO28 and nonadapted ATM56 (□) cultures at pH 3.5 is shown. Error bars represent the standard deviation for triplicate experiments.

and whole-fat and low-fat cheddar cheeses were obtained from the National Dairy Products Research Centre, Fermoy, Ireland. Other foods which were obtained locally from retail outlets included yogurt, cottage cheese, salad dressing, and orange juice.

**Adaptation of *L. monocytogenes* to acid and measurement of ATR.** An overnight culture of *L. monocytogenes* grown in TSB-YE was used to inoculate fresh broth. Cells were grown statically to an  $A_{600}$  of 0.15 (early log phase). Duplicate samples were centrifuged and resuspended in TSB-YE adjusted with 1 M lactic acid to pH 5.5 (adapted) or pH 7.0 (nonadapted). Following incubation at 37°C for 60 min, the cells were harvested by centrifugation and resuspended in TSB-YE acidified to pH 3.5 with 3 M lactic acid (challenge pH). These acidified cultures were incubated for up to 120 min at 37°C, and viable plate counts were performed at intervals by serial dilution of samples in quarter-strength Ringer's solution and plating onto tryptone soy agar (TSA) plates containing 0.6% yeast extract. The effectiveness of the ATR phenomenon in protecting cells from low pH is shown in Fig. 1.

The isolation of the acid-tolerant mutant, designated ATM56, has been described previously (24). Briefly, an overnight culture of *L. monocytogenes* LO28 was adjusted to pH 3.5, and survivors were isolated following prolonged exposure (18 h) at 37°C. Individual survivors were grown in TSB-YE at pH 7.0 and subsequently subjected to repeated screening for increased tolerance to lethal pH. In this manner, a number of mutants, including ATM56, which are constitutively resistant to low pH in the absence of adaptation, were isolated (Fig. 1).

Prior to inoculation of foods, acid-adapted and nonadapted *L. monocytogenes* LO28 and ATM56 were washed once with quarter-strength Ringer's solution, centrifuged, and resuspended in 1 ml of quarter-strength Ringer's solution.

**Survival of *L. monocytogenes* in cottage cheese.** Cottage cheese (pH 4.71) was prepared with a 0.02% inoculum of L253 deep-frozen lactic acid starter culture in 2 liters of sterile skim milk. A short-set production procedure was used (32°C for 5 to 6 h) (17). Fermentation was allowed to continue until the target pH was achieved. Cheese portions were weighed aseptically into sterilized containers.

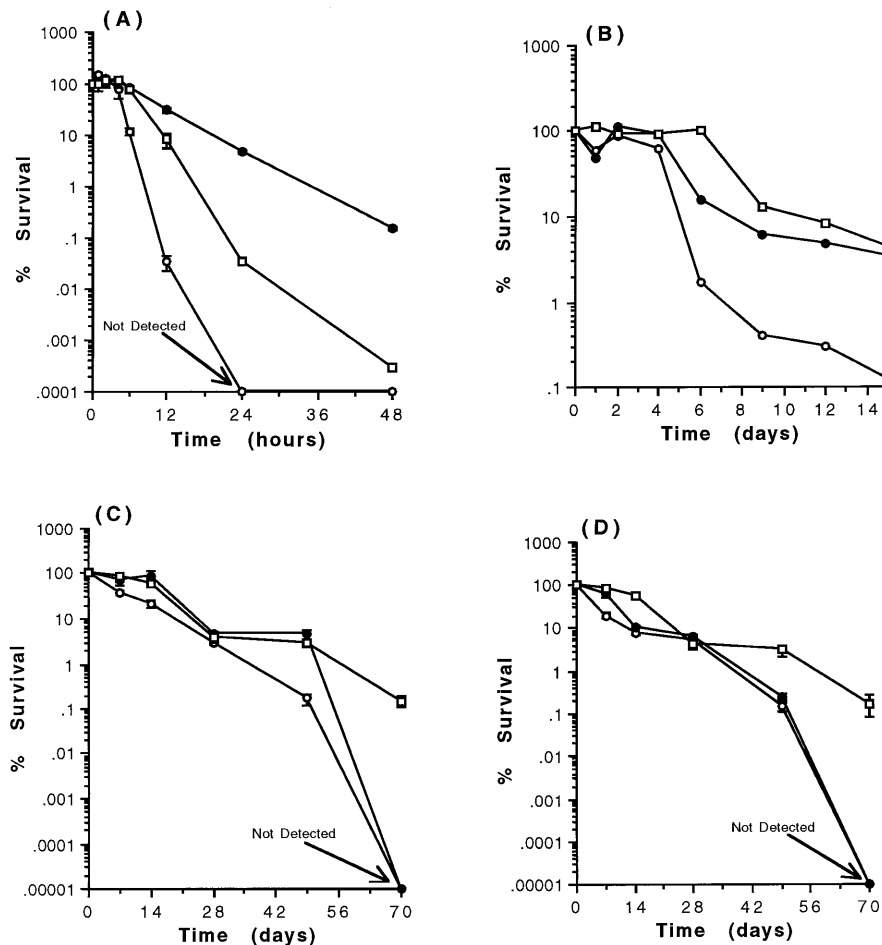


FIG. 2. Survival of *L. monocytogenes* during storage and ripening of dairy products. Symbols: ●, acid-adapted *L. monocytogenes* LO28; ○, nonadapted *L. monocytogenes* LO28; □, nonadapted ATM56. The dairy products tested were natural yogurt (pH 3.9) (A), cottage cheese (pH 4.71) (B), whole-fat cheddar cheese (pH 5.16) (C), and low-fat cheddar cheese (pH 5.25) (D). Error bars represent the standard deviation for duplicate (A) or triplicate (C and D) experiments.

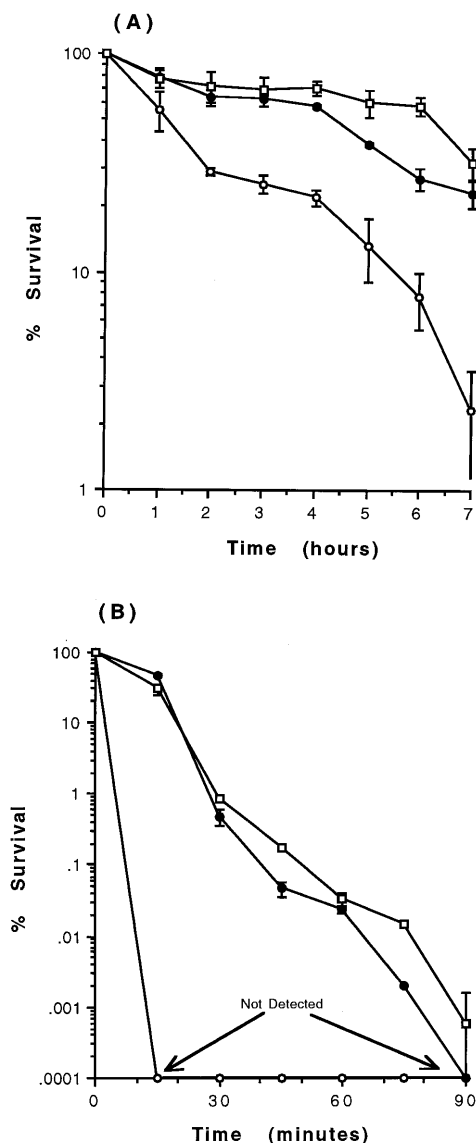


FIG. 3. Survival of *L. monocytogenes* in orange juice (pH 3.76) (A) and salad dressing (pH 3.0) (B). Symbols: ●, acid-adapted *L. monocytogenes* LO28; ○, nonadapted *L. monocytogenes* LO28; □, nonadapted ATM56. Error bars represent the standard deviation for duplicate experiments.

LO28 (acid adapted or nonadapted) or ATM56 was inoculated and mixed thoroughly to give an initial concentration of  $10^5$  cells per g. The spiked samples were stored at 4°C and tested at time intervals up to 15 days. *L. monocytogenes* counts were determined by spread plating onto LSA plates. Survival of *Listeria* cultures in a commercially obtained cottage cheese was enumerated by the same procedure.

**Survival of *L. monocytogenes* in hard cheeses.** Whole-fat cheddar (pH 5.16), low-fat cheddar (pH 5.25), and mozzarella (pH 5.6) cheeses were divided into 50-g blocks, which were subsequently sliced in half. Aliquots (100  $\mu$ l) of LO28 (acid adapted or nonadapted) or ATM56 were inoculated onto the freshly exposed surface of each piece of cheese. The two halves were then closed to give a sandwich effect. The initial concentration of cells was ca.  $10^3$  to  $10^4$  cells per g of cheese. Each piece of cheese was placed inside a sterile seal bag and vacuum packaged. The cheddar cheese was incubated at 8°C (ripening temperature for cheddar), and the mozzarella cheese was incubated at 4°C (ripening temperature for mozzarella). Each sample was aseptically homogenized in 2% trisodium citrate solution in a Colworth stomacher for 5 to 10 min. Subsequently, the homogenate was serially diluted in quarter-strength Ringer's solution, and appropriate dilutions were used to enumerate survivors on LSA plates.

**Survival of *L. monocytogenes* in other low-pH foods.** A commercial salad dressing (pH 3.0) and orange juice (pH 3.76) were inoculated with LO28 (acid

adapted or nonadapted) or ATM56 to an initial concentration of  $10^6$  cells per ml. Both samples were incubated at 4°C. Following appropriate time intervals, samples were taken and survivors were enumerated by plating serial dilutions onto LSA plates.

**Survival of *L. monocytogenes* in a natural yogurt and during active milk fermentation.** Activated *S. thermophilus* starter culture (1 ml) was inoculated into 50 ml of sterile skim milk (pH 6.7) at 37°C, and fermentation was allowed to proceed until either pH 5.5 or 4.85 was reached. Acid-adapted or nonadapted *L. monocytogenes* LO28 or ATM56 was then inoculated to give an initial concentration of ca.  $10^5$  cells per ml. Survivors were enumerated after various time intervals by serial dilution in quarter-strength Ringer's solution and plated onto LSA plates. A commercially produced natural yogurt (pH 3.9) was inoculated with *L. monocytogenes* to similar levels but was incubated at 4°C.

**Statistical methods.** Individual experiments were performed in duplicate or triplicate as stated in the figure legends. Datum points are represented by the mean, with the standard deviation indicated by error bars. Data sets at particular time points were subjected to a Student *t* test.

## RESULTS

**Survival of adapted and nonadapted *L. monocytogenes* in acidified dairy products.** We have evaluated the influence of acid tolerance, as a consequence of either adaptation or mutation, on the survival of *L. monocytogenes* in a number of acidified dairy products. A commercially available natural yogurt (pH 3.90) and a cottage cheese manufactured in the laboratory (pH 4.71) were inoculated with acid-adapted or nonadapted *L. monocytogenes* LO28 or with the acid-tolerant mutant ATM56. Both ATM56 and the acid-adapted *L. monocytogenes* LO28 demonstrated greatly enhanced survival in these low-pH foods compared with the nonadapted wild type. In yogurt, increased acid resistance influenced survival to the extent that both acid-adapted LO28 and the constitutively tolerant ATM56 could be detected 48 h postinoculation (Fig. 2A). In contrast, nonadapted *L. monocytogenes* LO28 was rapidly inactivated in yogurt and could not be detected at 24 or 48 h following inoculation. The adapted LO28 showed the greatest survival rate, significantly higher than that of the acid-tolerant mutant ATM56 ( $P > 0.05$ ). Similarly, acid tolerance enhanced the long-term survival of *L. monocytogenes* cultures in cottage cheese, with ATM56 and the acid-adapted LO28 surviving in greater numbers than the nonadapted LO28 (Fig. 2B). In this instance, the mutant, ATM56, fared as well as the adapted LO28 in terms of survival rate. In contrast, when the same cultures were inoculated into a commercial cottage cheese (pH 5.15), all strains, including nonadapted LO28, survived well during a 26-day period of study (data not shown). It is possible that the commercial cottage cheese products, which have been dressed with nonacidified cream, do not provide a sufficiently acidic environment to allow a selective advantage for acid-resistant cultures.

The influence of acid tolerance on the survival of *Listeria* cells during ripening of hard cheeses was also studied. Whole-fat and low-fat cheddar cheeses were sandwich inoculated with LO28 (acid adapted or nonadapted) or ATM56. Acid adaptation marginally increased the survival of LO28 in whole-fat cheddar cheese (pH 5.16) but was not sufficient to allow the strain to survive a 70-day ripening period (Fig. 2C). Similarly, acid-adapted LO28 did not exhibit enhanced survival during the ripening of low-fat cheddar cheese (pH 5.25) and did not survive the ripening period (Fig. 2D). However, the acid-tolerant mutant ATM56 displayed greater resistance during the ripening of both low- and whole-fat cheddars, with significant numbers recovered after 70 days.

Additional experiments conducted at 4°C with unripened mozzarella (pH 5.60) also revealed no enhanced survival due to acid adaptation (data not shown). In this case, no significant reduction in the numbers of acid-adapted and nonadapted LO28 and ATM56 was seen over a 4-week ripening period.

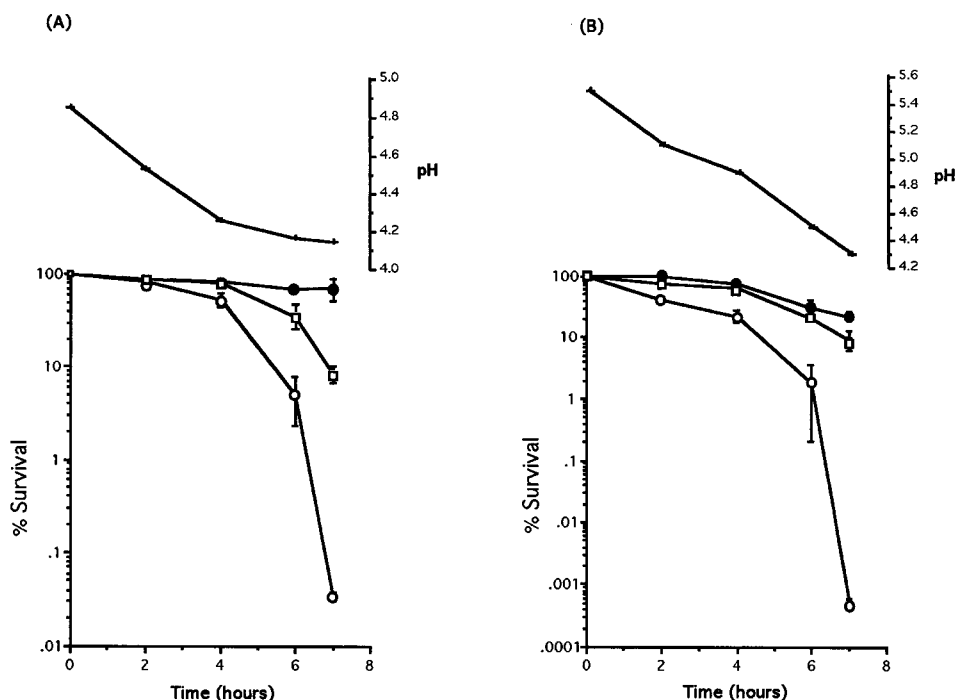


FIG. 4. Survival of *L. monocytogenes* during active milk fermentation by *S. thermophilus*. Symbols: ●, acid-adapted *L. monocytogenes* LO28; ○, nonadapted *L. monocytogenes* LO28; □, nonadapted ATM56. *L. monocytogenes* cultures were added at pH 4.8 (A) or pH 5.5 (B). Error bars indicate the standard deviation for duplicate experiments. Datum points with no visible error bars indicate deviations smaller than the symbol.

**Survival of *L. monocytogenes* in other low-pH foods.** We have previously determined that induction of the ATR in *L. monocytogenes* can enhance microbial survival in the presence of a number of acids (24). To determine the inhibitory properties of various food-grade acidulents against *L. monocytogenes*, we investigated the survival of acid-adapted and nonadapted *Listeria* cultures in commercially available orange juice (citric acid [pH 3.76]) and salad dressing (acetic acid [pH 3.0]). The overall survival rate was higher in the orange juice than the salad dressing, with approximately 2% of the noninduced LO28 cells surviving after 7 h (Fig. 3A). This compares with approximately 30 and 40% survival of the adapted strain and the mutant, respectively. The cells in the salad dressing died more rapidly, with no nonadapted cells surviving after 15 min. Once again, the adapted and mutant strains displayed a much reduced death rate with respect to the parent (Fig. 3B). The data demonstrate that induction of acid tolerance in *L. monocytogenes* can influence the survival of the pathogen not only in certain dairy products but also in other low-pH foods.

**Survival of *L. monocytogenes* during milk fermentation.** We examined the effect of the ATR on survival of *L. monocytogenes* during active milk fermentation by *S. thermophilus*. The *S. thermophilus* strain used in this study does not produce a bacteriocin that is active against *L. monocytogenes*. Acid-adapted or nonadapted *L. monocytogenes* cultures were added when the fermentation reached pH 4.8. The subsequent survival of acid-adapted *L. monocytogenes* cells was greatly enhanced compared with that of the nonadapted culture, especially during the later stages of fermentation (Fig. 4A). Nonadapted *L. monocytogenes* cells were rapidly inactivated, whereas survival of the acid-adapted culture was greatly enhanced. Seven hours following *L. monocytogenes* inoculation, the fermentation reached pH 4.15. At this stage, there was a 3-log-unit difference between the survival of acid-adapted and

nonadapted *L. monocytogenes* cultures. It is also notable that the acid-tolerant mutant, ATM56, survived as well as the acid-adapted culture during the first 4 h of the milk fermentation before exhibiting a marked decrease in survival toward the end of the fermentation (Fig. 4A). The data indicate that either innate acid tolerance or induction of an ATR in *L. monocytogenes* can greatly enhance survival of the pathogen during milk fermentation.

Similar results were obtained when *L. monocytogenes* cultures were added to the fermentation at pH 5.5 (Fig. 4B). In this situation, nonadapted *L. monocytogenes* LO28 remained relatively acid sensitive as the pH conditions became lethal. This indicates that acid tolerance is not fully induced in log-phase *Listeria* cultures during milk fermentation. The ability of the pathogen to adapt to mildly acidic conditions in foods requires further study.

## DISCUSSION

A number of studies have confirmed that the low pH of some foods is an important factor in preventing the growth of *L. monocytogenes* and other food-borne pathogens (1, 9, 11, 15). In nature, it is likely that adaptive tolerance responses to acidity, salt, and suboptimal growth temperatures can permit bacterial survival in environments that are lethal for nonadapted cells. Many bacteria, including *L. monocytogenes*, can acquire increased resistance to low pH following exposure to sublethal acidic conditions (6, 10, 13, 14, 16, 24). This physiological response to mildly acidic stress is designated the ATR and has the potential to influence the survival of bacteria in acidic environments which are suboptimal for growth. Adaptation of *E. coli* and *Salmonella* spp. to mildly acidic conditions significantly increases their survival in foods compared with that of nonadapted cultures (19, 20). Here we demonstrate

that the survival of *L. monocytogenes* in low-pH foods can be similarly enhanced if the bacterium is acid adapted prior to inoculation. In addition, we show that an acid-tolerant mutant of *L. monocytogenes* is capable of enhanced survival in a number of acidic foods.

In this study, we demonstrate the enhanced survival of acid-adapted *L. monocytogenes* strains in foods containing lactic acid (yogurt [pH 3.87] and cottage cheese [pH 4.71]), citric acid (orange juice [pH 3.65]), or acetic acid (salad dressing [pH 3.0]). Previous studies in our laboratory have shown that induction of the ATR in *Listeria* cells provides a degree of cross-protection against other stresses such as osmotic shock and heat shock (24). It has also been shown that application of sublethal stresses can increase the resistance of *L. monocytogenes* to subsequent stresses (2, 4, 21). Therefore, it is possible that the enhanced survival of acid-adapted *L. monocytogenes* will reflect the induction of a general stress-resistant phenotype rather than specific protection against acid stress alone.

The increased survival of the acid-adapted *Listeria* strains has important consequences for food processors with a responsibility for ensuring the safety of consumers. For example, the survival of the adapted and mutant strains for a period of 2 weeks in low-pH cottage cheese and the survival of the mutant strain for 70 days in both whole-fat and low-fat cheddar cheeses may have profound implications for cheesemakers. In addition, our data demonstrate that the survival of *L. monocytogenes* during fermentation of milk by lactic acid bacteria is greatly enhanced by acid adaptation prior to inoculation. There was a correlation between enhanced survival and the pH of the milk, with adapted cells surviving significantly better than nonadapted cells when the pH fell below 4.4. It is interesting that nonadapted *Listeria* cultures failed to become fully adapted to acidic conditions during fermentation even when added at an inducing pH (pH 5.5).

We have previously described the isolation of acid-tolerant mutants of *L. monocytogenes* (13, 24), and acid-tolerant mutants of *S. typhimurium* have also been reported (6). In the present study, an acid-tolerant mutant of *L. monocytogenes* demonstrated greater survival in low-pH foods than did the nonadapted wild-type strain. During ripening of both whole-fat and low-fat cheddar cheese, survival of this acid-tolerant mutant was significantly enhanced compared with that of the parent. Work is under way in our laboratory to determine whether *Listeria* strains with increased natural acid resistance exist in nature.

Collectively, our data demonstrate that prior adaptation of *L. monocytogenes* to mildly acidic conditions can greatly enhance the survival of the pathogen in low-pH foods. The results are in full agreement with those of similar studies examining the survival of gram-negative food-borne pathogens in a range of acidic foods (19, 20). Although low-pH foods have not been implicated as a vehicle for transmission of listeriosis, our data indicate that adaptive stress responses must be taken into consideration when establishing protocols for the inactivation of *L. monocytogenes* in foods.

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