APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1997, p. 4945–4947 Vol. 63, No. 12
0099-2240/97/$04.00+0

A Mutant of *Listeria monocytogenes* LO28 Unable To Induce an Acid Tolerance Response Displays Diminished Virulence in a Murine Model

LYNDA MARRON,[1] NATHAN EMERSON,[1] CORMAC G. M. GAHAN,[1,2] AND COLIN HILL[1,2]*

Microbiology Department[1] and The National Food Biotechnology Centre,[2] University College Cork, Cork, Ireland

Received 27 June 1997/Accepted 25 August 1997

Exposing *Listeria monocytogenes* LO28 to sublethal pH induces protection against normally lethal pH conditions, a phenomenon known as the acid tolerance response. We identified a mutant, *L. monocytogenes* ATR1, which is incapable of inducing such tolerance, either against low pH or against any other stress tested. The virulence of this mutant was considerably decreased, suggesting that the acid tolerance response contributes to in vivo survival of *L. monocytogenes*.

Listeria monocytogenes encounters a variety of stressful conditions during infection of an animal host. It is likely that the ability of *L. monocytogenes* to sense and respond to changes in its surroundings is crucial to its survival. One such survival response is termed the acid tolerance response (ATR), a phenomenon which has profound implications for the survival of *L. monocytogenes* in low-pH foods (3) and potentially in the acidic environments of the stomach and the macrophage phagosome. The ATR is typically demonstrated by the enhanced survival of *L. monocytogenes* at a normally lethal pH following adaptation to mildly acidic conditions (2, 4). We have previously reported that an acid-tolerant mutant of *L. monocytogenes* demonstrated increased virulence in a mouse model (4). This report confirms that the following corollary is also true: a mutant of *L. monocytogenes* which is incapable of mounting an ATR displays reduced virulence, confirming the importance of this inducible response during infection.

Isolation of *L. monocytogenes* mutant ATR1. In order to detect *Listeria* genes whose transcription is influenced by a shift to acidic growth conditions, a Tn*917-lac* bank of *L. monocytogenes* LO28 was constructed by using a previously described method (1, 5). When this system is used, insertion of the transposon into regions that are expressed under specified growth conditions gives rise to a Lac^+ phenotype, which is indicated by blue colonies on plates containing the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Transposon insertions into regions that are not expressed give rise to white colonies. More than 1,500 individual colonies were replica plated onto plates containing tryptone soy agar (Sigma Chemical Co., St. Louis, Mo.) supplemented with 0.6% yeast extract (TSA-YE) containing X-Gal (80 μg/ml) at pH 7.2 and onto similar plates adjusted to pH 6.0. Six isolates were identified which gave rise to white colonies at pH 7.2, but formed blue colonies at pH 6.0. Five of these isolates also produced blue colonies on plates containing 3.0% NaCl or plates incubated at 42°C. The remaining isolate, designated ATR1, reacted only to a change in pH. Upon initial subculturing, ATR1 lost the ability to produce a blue color on TSA-YE containing X-Gal adjusted to pH 6.0 and remained white under all conditions. PCR assays performed with a number of Tn*917*-specific primers failed to demonstrate the presence of the transposon in the host, and this was subsequently confirmed by using plasmid pLTV1 (5) as a probe in Southern hybridizations (data not shown). The combined evidence suggests that the entire transposon was deleted from the ATR1 genome. However, we assume that the deletion event was imprecise, given that a mutant phenotype remains even in the absence of the transposon.

ATR1 is incapable of a log-phase ATR. The ability of ATR1 to induce an ATR was examined as follows. Overnight cultures of *L. monocytogenes* were grown statically to an A_{600} of 0.15 (early log phase). Duplicate samples were harvested and resuspended in either TSB-YE adjusted to pH 5.5 with 3 M lactic acid (adapted) or TSB-YE at pH 7.0 (nonadapted). Following incubation at 37°C for 60 min, 100 μl of each sample was inoculated into 10 ml of TSB-YE adjusted to pH 3.5 (challenge pH) with 3 M lactic acid. Survival over 2 h at 37°C was determined by performing plate counts at 30-min intervals on TSA-YE (Fig. 1A). Noninduced ATR1 demonstrated sensitivity to the challenge pH similar to the sensitivity of noninduced LO28, but induced ATR1 displayed no enhanced tolerance. Thus, we concluded that ATR1 is not able to adapt to become tolerant to low pH.

It is well established that the growth phase influences the degree of acid resistance displayed by *L. monocytogenes* (2, 4). An overnight culture of ATR1 was inoculated into fresh TSB-YE and incubated at 37°C for 1 h. The initial number and the percentage of survival at each time point were determined by determining viable plate counts on TSA-YE (Fig. 1B). ATR1 displays a growth phase-dependent ATR similar to that shown previously for parent strain LO28 (4). Thus, while the strain cannot induce acid tolerance in response to log-phase induction at pH 5.5, it is capable of stationary-phase tolerance. This does not necessarily suggest that stationary-phase tolerance and inducible tolerance are distinct mechanisms, but the data may simply reflect the fact that there is a single mechanism that can be triggered by different signals. The mutation in ATR1 presumably affects the ability of this organism to respond to the induction pH, rather than representing a deficiency in a key acid tolerance gene.

Cross-protection against other stresses and the heat shock response. Acid-adapted LO28 has been shown to exhibit cross-protection against other stresses (4). The ability of ATR1 to induce such cross-protection was investigated. Log-phase cul-

* Corresponding author. Mailing address: Microbiology Department, University College Cork, Cork, Ireland. Phone: 353-21-902397. Fax: 353-21-903101. E-mail: c.hill@ucc.ie.

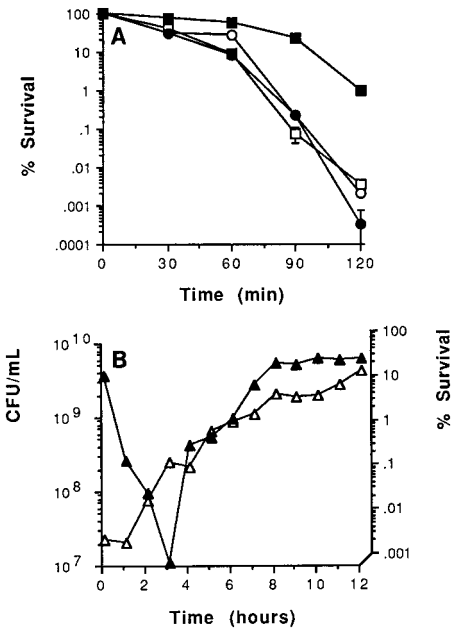

FIG. 1. (A) ATR in *L. monocytogenes* LO28 and ATR1. The survival of acid-adapted LO28 (■) and ATR1 (●) and nonadapted LO28 (□) and ATR1 (○) cultures at pH 3.5 is shown. Error bars represent the standard deviations from triplicate experiments. (B) Growth phase-dependent acid sensitivity of *L. monocytogenes* ATR1. The numbers of CFU were estimated at various stages of growth (△). Percentages of survival were simultaneously determined after exposure to pH 3.5 for 60 min (▲). Data are representative of the data from triplicate experiments.

tures of *L. monocytogenes* LO28 and ATR1 were exposed to pH 5.5 (adapted) or pH 7.0 (nonadapted) as described above. The ability of these cultures to survive thermal stress was examined in TSB-YE at 54°C for up to 120 min (Fig. 2A). Survival in TSB-YE containing 15% ethanol (Fig. 2B) or 10 mg

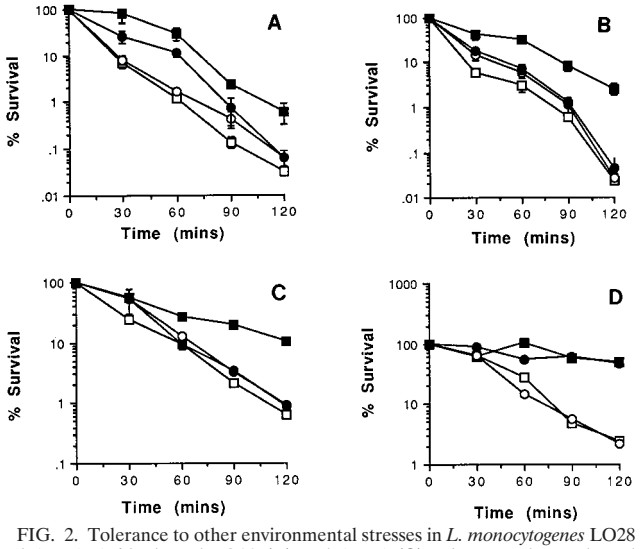

FIG. 2. Tolerance to other environmental stresses in *L. monocytogenes* LO28 and ATR1. Acid-adapted LO28 (■) and ATR1 (●) cultures and nonadapted LO28 (□) and ATR1 (○) cultures were exposed to 54°C (A), 15% ethanol (B), and 10 mg of crystal violet per ml (C). Alternatively, heat-adapted LO28 (■) and ATR1 (●) cultures and nonadapted LO28 (□) and ATR1 (○) cultures were exposed to 54°C (D). Error bars represent the standard deviations from triplicate experiments.

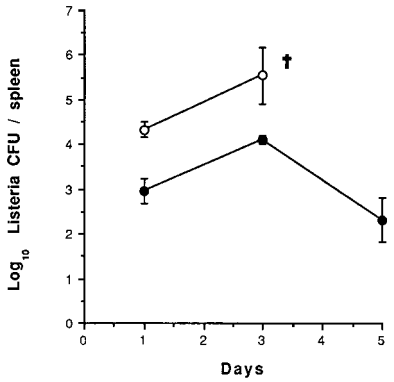

FIG. 3. Growth rates of *L. monocytogenes* LO28 (○) and ATR1 (●) in spleens of BALB/c mice. Mice were inoculated with 3×10^6 *Listeria* cells by the intraperitoneal route. The cross indicates that mice were dead after day 3. Each datum point represents the mean $\log_{10}$ number of viable *Listeria* cells per spleen for four mice (± standard deviation [error bars]).

of crystal violet per ml (Fig. 2C) was also examined. Briefly, *L. monocytogenes* ATR1 which was subjected to acid adaptation conditions did not show any enhanced resistance to any of the stresses, whereas a significant degree of protection was conferred on LO28.

In order to examine the ability of *L. monocytogenes* ATR1 to induce a heat shock response, log-phase cultures were centrifuged in TSB-YE preheated to either 42°C (adapted) or 37°C (nonadapted). The cultures were incubated at the relevant temperatures for 20 min and then challenged by inoculation into TSB-YE and incubation at 54°C for up to 120 min (Fig. 2D). Although adaptation in acid did not provide protection against exposure to high temperature (Fig. 2A), *L. monocytogenes* ATR1 is capable of inducing a heat shock response (Fig. 2D). Thus, the mutation in ATR1 can be said to be specific in its ability to respond to low pH and is not a defect in a general stress pathway.

ATR1 has diminished virulence. We have previously shown that an acid-tolerant mutant of LO28 displays enhanced virulence compared with the parental strain (4). Thus, we were interested to determine whether the ability to induce an ATR could be considered a virulence factor. Bacterial virulence was assessed by monitoring the ability of *L. monocytogenes* ATR1 and LO28 to reach and grow in the spleens of infected mice. Eight- to twelve-week-old BALB/c mice were inoculated intraperitoneally with 3×10^6 cells suspended in 0.2 ml of phosphate-buffered saline (containing [per liter] 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4, 0.2 g of KCl, 8.0 g of NaCl; pH 7.2). Mice were sacrificed over a 5-day period following infection, and the numbers of *Listeria* cells in the spleens were determined by plating organ homogenates onto TSA-YE. Considerably fewer *L. monocytogenes* ATR1 cells than parental strain LO28 cells were detected in the spleens on day 1. However, those ATR1 cells which reached the spleens had a growth rate similar to that of the parent cells until day 3, after which the mice infected with LO28 died, while the mice infected with ATR1 cleared the infection (Fig. 3). The genetic defect in ATR1 resulted in an increased lethal dose for this strain since the dose used was lethal with parent strain LO28 but not with the mutant. For comparison, Bof415 (a hemolysin-negative derivative of LO28 and a gift from P. Cossart, Pasteur Institute, Paris, France) was found to be completely avirulent in this model and was not detected in the spleens of infected mice (data not shown). It should be noted that ATR1 is unaffected

in its ability to produce both hemolysin and lecithinase, indicating that the primary virulence genes have not been disrupted (data not shown). The ability to adapt to low pH seems, therefore, to be an important factor contributing to the virulence of *L. monocytogenes.*

The precise defect in mutant ATR1 is not known. However, this mutant shows that the ability to adapt to acid stress is important for the pathogenesis of *L. monocytogenes*. A similar study has shown that mutation of the molecular chaperone ClpC in *Listeria* cells also results in a reduction in virulence (6). However, PCR analysis of ATR1 revealed no detectable alteration of the ClpC operon. Work to further characterize this mutant is continuing in our laboratory. We hope to gain a more precise understanding of the mechanisms by which the ATR and other stress responses are controlled and of the role played by stress responses in the virulence of *L. monocytogenes.*

We acknowledge the support of the Non-Commissioned Food Research Programme, operated by the Irish Department of Agriculture, Food and Forestry and supported by national and European Union funds.

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