

Listeria monocytogenes ArcA contributes to acid tolerance

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The foodborne pathogen *Listeria monocytogenes* is able to colonize the human and animal intestinal tracts and subsequently crosses the intestinal barrier, causing systemic infection. For successful establishment of infection, *L. monocytogenes* must survive and adapt to the low pH environment of the stomach. Gene sequence analysis indicates that *Imo0043*, an orthologue of *arcA*, encodes a protein containing conserved motifs and critical active amino acids characteristic of arginine deiminase that mediates an arginine deimination reaction. We attempted to characterize the role of ArcA in acid tolerance *in vitro* and in mice models. Transcription of *arcA* was significantly increased in *L. monocytogenes* culture subjected to acid stress at pH 4.8, as compared with that at pH 7.0. Deletion of *arcA* impaired growth of *L. monocytogenes* under mild acidic conditions at pH 5.5, and reduced its survival in synthetic human gastric fluid at pH 2.5 and in the murine stomach. Bacterial load in the spleen of mice intraperitoneally inoculated with an *arcA* deletion mutant was significantly lower than that of the wild-type strain. These phenotypic changes were recoverable by genetic complementation. Thus, we conclude that *L. monocytogenes arcA* not only mediates acid tolerance *in vitro* but also participates in gastric survival and virulence in mice.

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INTRODUCTION

The intracellular foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a severe invasive illness of high mortality (Mead *et al.*, 1999). Foodborne transmission is the most common means of contracting both epidemic and sporadic listeriosis, with 99 % of human cases caused by consumption of contaminated food products (Chen *et al.*, 2009a). *L. monocytogenes* encounters acid stresses in a number of stages during the course of its infectious cycle, from food to the gastrointestinal tract and in the phagosomes of macrophages (Cotter & Hill, 2003; Gray *et al.*, 2006). *L. monocytogenes* possesses a number of potential mechanisms for pH homeostasis to survive and proliferate to high numbers under acidic conditions, which could be considered as a virulence mechanism (Chen *et al.*, 2011; Cotter *et al.*, 1999; Marron *et al.*, 1997; Merrell & Camilli, 2002; O'Driscoll *et al.*, 1996; Wiedmann *et al.*, 1998). The glutamate decarboxylase (GAD) system, which consumes intracellular protons by converting glutamate to

γ -aminobutyrate (Cotter *et al.*, 2001a), has been suggested as an acid-resistance system of *L. monocytogenes* for its survival in low pH foods (Cotter *et al.*, 2001b; Gahan *et al.*, 1996). *L. monocytogenes* also has the F₁F₀-ATPases system, which plays an important role in pH homeostasis by using the ATP generated from arginine to extrude cytoplasmic protons (Cotter *et al.*, 2000).

The arginine deiminase (ADI) pathway has been found to play an important role in the survival of several bacterial species such as *Streptococcus pyogenes*, *Streptococcus suis* and *Lactobacillus brevis* under acidic conditions *in vitro* (Degnan *et al.*, 2000; Fulde *et al.*, 2011; Gruening *et al.*, 2006; Lucas *et al.*, 2007; Marquis *et al.*, 1987; Vrancken *et al.*, 2009). The ADI pathway involves hydrolysis of arginine to citrulline and ammonia, and the generation of ATP molecules by three enzymes, namely ADI (EC 3.5.3.6), ornithine transcarbamylase (EC 2.1.3.3) and carbamate kinase (EC 2.7.2.2) encoded by the *arcA*, *arcB* and *arcC* genes, respectively (Cunin *et al.*, 1986). Ammonia produced from the system combines with intracellular protons to yield NH₄⁺ and this reaction raises the cytoplasmic pH,

Abbreviations: ADI, arginine deiminase; GAD, glutamate decarboxylase.

thereby protecting the cell from potentially lethal effects of acidic extracellular environments.

Homologues of the ADI system genes have been found in the sequenced genome of *L. monocytogenes* strains by *in silico* analysis (Glaser *et al.*, 2001). ADI encoded by *lmo0043*, an orthologue of *arcA*, is the key enzyme in the ADI pathway which initiates the first reaction. Ryan *et al.* (2009) reported molecular characteristics of the ADI system and its *in vitro* contributions to acid tolerance in *L. monocytogenes*. However, better understanding of the role of ADI in the acid stress response *in vivo* and potential pathogenicity of *L. monocytogenes* is still lacking. In this study, we used gene knockout and genetic complementation approaches to characterize the gastric survival and virulence of *L. monocytogenes* in mouse models.

METHODS

Bacterial strains, plasmids and culture conditions. *L. monocytogenes* reference strain 10403S was used as the wild-type strain. *Escherichia coli* DH5 α was employed as the host strain for plasmids pMD18-T (Takara), pERL3 (Chen *et al.*, 2011) and pKSV7 (Wiedmann *et al.*, 1998; Chen *et al.*, 2009b). *L. monocytogenes* wild-type and mutant strains were cultured in brain heart infusion (BHI; Oxoid) medium and *E. coli* strains were grown in Luria–Bertani medium (Oxoid). Chloramphenicol and erythromycin were used, where appropriate, at final concentrations of 10 and 5 $\mu\text{g ml}^{-1}$, respectively, for *L. monocytogenes*, and ampicillin, erythromycin and chloramphenicol were used at final concentrations of 50, 150 and 50 $\mu\text{g ml}^{-1}$, respectively, for *E. coli*.

Bioinformatic analysis. Amino acid sequences of *arcA* of *L. monocytogenes* and its orthologues in other microbial species were obtained from the National Center for Biotechnology information (<http://www.ncbi.nlm.nih.gov/>). The known crystal structures and active sites of ArcA were acquired from the Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>). These amino acid sequences were aligned using DNASTAR software.

Construction of the *arcA* deletion mutant. A homologous recombination strategy was used for the in-frame deletion of the

full-length *arcA* (1233 bp) from *L. monocytogenes* 10403S according to the protocol described previously (Chen *et al.*, 2009b; Monk *et al.*, 2008). Primer pairs *arcA-a/arcA-b* and *arcA-c/arcA-d* were used to generate fragments of 518 and 559 bp from flanking regions of *arcA* (Table 1). The resultant products were purified using an AxyPrep DNA Gel Extraction kit (Axygen) and were used for the next round of amplification using external primer pair *arcA-a/arcA-d*. The final product containing *Hind*III and *Kpn*I (Takara) sites was digested and ligated to pMD18-T vector (Takara). After sequencing confirmation, the inserted fragment was subcloned into the temperature-sensitive shuttle vector pKSV7 and transformed into *E. coli* DH5 α . Recombinant plasmids were subsequently extracted and electroporated into the competent *L. monocytogenes* 10403S cells (Monk *et al.*, 2008). Transformants were grown at a non-permissive temperature (41 °C) in BHI medium containing chloramphenicol to promote chromosomal integration and the recombinants were passaged successively in BHI medium without antibiotic at a permissive temperature (30 °C) to enable plasmid excision and curing (Camilli *et al.*, 1993). The recombinants were identified as chloramphenicol-sensitive colonies and confirmed by PCR with the *arcA-e/arcA-f* primers, which targeted the chromosomal regions upstream or downstream of the homologous arms. The resultant knockout mutant was designated Δ arcA.

Complementation of the *arcA* deletion. The *arcA* complementation was conducted according to a previous protocol (Chen *et al.*, 2011). The *arcA* ORF and its upstream promoter region were amplified from 10403S using primer pair *arcA-pERL3-F/arcA-pERL3-R* (Table 1). The product containing *Sac*I and *Bam*HI sites was digested, ligated to pMD18-T, and the sequence was confirmed and subcloned into the replicating vector pERL3. The resulting vector was then electroporated into the mutant strain Δ arcA, and positive transformants were verified by selecting on BHI plates containing erythromycin (5 $\mu\text{g ml}^{-1}$). The complemented mutant was designated C Δ arcA.

Analysis of ADI activity by citrulline determination. Citrulline, formed by ADI from arginine, could be quantified by colorimetric determination of its reaction product with diacetyl-monoxime, performed (with some modifications) as described by Knipp & Vasák (2000). The colouring reagent consisted of one volume of solution A and three volumes of solution B [solution A: 80 mM diacetyl-monoxime (Sigma-Aldrich) and 2.0 mM thiosemicarbazide (Sigma); solution B: 3 M H_3PO_4 , 6 M H_2SO_4 and 2 mM $\text{NH}_4\text{Fe}(\text{SO}_4)_2$]. The calibration curve for quantitative analysis of ADI activity was obtained by mixing 400 μl of the colouring reagent with 120 μl of different

Table 1. PCR primers used in this study

Nucleotides introduced to create restriction sites are underlined.

| Primer name | Primer sequence (5'–3') | Product size (bp) |
|--------------|-----------------------------------|-------------------|
| arcA-a | ATAAGCTTCTTTATGGTTAGCAGCAGTTC | 518 |
| arcA-b | CTCATTTTCAAAGCCACCTCTTCCTA | |
| arcA-c | GCTTTGAAAAATGAGAATCCCCTTTC | 559 |
| arcA-d | TAGGTACCCATATGACGAATGATATCATC | |
| arcA-e | GAGACTGGATTGGTAGTATTTCCAT | 2511 |
| arcA-f | CGTCCTACAAGTACTACACGATTCA | |
| arcA-pERL3-F | CGCGAGCTCTCTGTGCTTTTTTTTATTT | 1312 |
| arcA-pERL3-R | CACGGATCCCTATTTAGATTTTCTCTAACAAGC | |
| arcA-RT-F | TGCGGAGGTTTGGATGAG | 95 |
| arcA-RT-R | TTGCCCATCATTTCCACTG | |
| gyrB-RT-F | AGACGCTATTGATGCCGATGA | 91 |
| gyrB-RT-R | GTATTGCGCGTTGTCTTCGA | |

concentrations of L-citrulline (0, 25, 50, 100, 200 and 400 μ M; Sigma). The mixtures were incubated at 95 °C for 15 min and were then cooled for 10 min at room temperature. Absorbance was measured at $A_{530\text{ nm}}$.

The cell-free supernatants for the ADI activity assay were prepared according to Ryan *et al.* (2009) by Triton X-100-based lysis (2 % Triton X-100, 100 mM NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA) of *L. monocytogenes* 10403S wild-type, mutant and complement strains grown overnight in BHI medium at pH 4.8 and 37 °C. The final supernatants (500 μ l) were added to 200 μ l 10 mM L-arginine (Sigma) in 100 mM potassium phosphate buffer (pH 6.5), and the reaction mixtures were incubated at 37 °C for 1 h. After incubation, 400 μ l colouring reagent was added to 120 μ l reaction samples, which were further incubated at 95 °C for 15 min. The amount of product reflecting ADI activity was quantified from the citrulline calibration curve.

Transcriptional analysis using quantitative PCR. *L. monocytogenes* wild-type 10403S, its mutant strain Δ arcA and complement strain C Δ arcA were grown to stationary phase (OD_{600} 0.6) in BHI broth (pH 7.0) at 37 °C, and they were then exposed to sublethal low pH (4.8) and neutral pH (7.0). After a 60 min exposure to acid stress, total RNA was prepared using the Trizol method and cDNA was synthesized using reverse transcriptase (Toyobo). Transcription of the *arcA* gene (primer pair *arcA*-RT-F/*arcA*-RT-R, Table 1) was quantified in 20 μ l reaction mixtures containing SYBR qPCR mix (Toyobo) on an iCycler iQ5 real-time PCR detection system (Bio-Rad). The housekeeping gene *gyrB* was selected as an internal control for normalization as previously described (Chen *et al.*, 2011). Total RNA was extracted from three separate cultures of each strain for qPCR. The transcriptional level of *arcA* at pH 7.0 was set as 100 % and results were expressed as mean \pm SD of three independent experiments.

Analysis of growth in acidic conditions. *L. monocytogenes* wild-type, mutant and complemented strains were grown overnight at 37 °C in BHI medium at pH 7.0 with shaking. The cultures were collected by centrifugation at 3000 g and 4 °C, washed in PBS (10 mM, pH 7.2), and the OD_{600} was adjusted to 0.6. The bacteria were then diluted 1 : 50 in 20 ml minimum essential medium (MEM; Gibco) (pre-adjusted to pH 5.5) or MEM at pH 7.0, and incubated at 37 °C. Growth was then measured at OD_{600} at appropriate intervals. The experiment was repeated three times, each in triplicate wells for each strain. This experiment was also performed in BHI medium at pH 5.5 and 7.0.

Survival in synthetic gastric fluid. *L. monocytogenes* wild-type, mutant and complemented strains were grown overnight at 37 °C in BHI medium at pH 7.0 with shaking. The cultures were collected by centrifugation at 3000 g at 4 °C and were washed and resuspended in PBS (10 mM, pH 7.2) with the OD_{600} adjusted to 0.6. One millilitre of the bacterial suspension was mixed in an equal volume of synthetic gastric fluid (per litre: 8.3 g proteose peptone, 3.5 g D-glucose, 2.05 g NaCl, 0.6 g KH_2PO_4 , 0.11 g $CaCl_2$, 0.37 g KCl, 0.05 g bile, 0.1 g lysozyme and 13.3 mg pepsin; adjusted to pH 2.5 with HCl) as described previously (Cotter *et al.*, 2001a; Werbrout *et al.*, 2008). After 30 min of incubation at 37 °C, the mixtures were serially diluted and plated onto BHI agar plates. The plates were incubated at 37 °C for 24 h and viable bacteria were counted. Percentage survival was reported as the mean \pm SD of three independent experiments, each performed in duplicate.

Survival in mouse gastrointestinal tracts. ICR mice (20 g, female) were divided into three groups (eight per group) and acclimatized for 3 days before experiments. Feed was removed 12 h prior to intragastric inoculation. Then, 200 μ l (4×10^8 c.f.u.) of *L. monocytogenes* wild-type, mutant and complemented strains grown overnight in BHI medium at pH 7.0 was administered to mice of corresponding groups by intragastric inoculation using a ball-tipped gavage needle. At 1 and 2 h after inoculation, the stomach samples

were separated and homogenized in sterile PBS (10 mM, pH 7.2). Serial dilutions of the homogenates were plated on PALCAM (*Listeria* selective medium; Luqiao) agar at 37 °C for 24 h for colony counting. The experimental settings ensured that animals were killed 1 or 2 h post-inoculation, and that organ homogenization and first dilution occurred 2.5 min after death; each phase was timed. Visible colonies were homologous and nine representative colonies (three from individual plates of each group) were confirmed by PCR as representing *L. monocytogenes* (Zeng *et al.*, 2006). A group of eight mice from the same batch was left uninoculated as controls and did not show visible colonies on PALCAM agar. The results were expressed as mean \pm SEM of \log_{10} c.f.u. per organ (stomach) for each group. All animal experiments in this study were approved by the Laboratory Animal Management Committee of Zhejiang University (Approval no. 20111025).

Virulence in the mouse model. The *L. monocytogenes* wild-type, mutant and complemented strains were tested for their recovery in the spleens of ICR mice (female, 20–22 g) as described previously (Chen *et al.*, 2009b). Groups of ICR mice (eight per group) were injected intraperitoneally with about 10^5 c.f.u. of each of the above bacterial strains. At 48 and 72 h post-infection, mice were killed. Spleens were removed and individually homogenized in PBS (10 mM, pH 7.2). Surviving *Listeria* cells were enumerated by plating serial dilutions of homogenates on BHI agar plates.

The LD₅₀ was determined by intraperitoneally inoculating serial dilutions of the *L. monocytogenes* wild-type and mutant strains into ICR mice (female, 20–22 g) as described by Hamon *et al.* (2006). The trimmed Spearman–Kärber method was used from the mortality data recorded over a 10 day period.

Survival in macrophage cell line. Survival in murine macrophage cells was conducted as described by O’Riordan *et al.* (2003). *L. monocytogenes* wild-type, mutant and complemented strains grown at 37 °C in BHI medium to stationary phase were washed and resuspended in PBS (pH 7.2). Murine RAW264.7 cells cultured to about 80 % confluency in Dulbecco’s modified Eagle medium (DMEM; Gibco) containing 10 % fetal calf serum (Gibco) were infected with the above strains for 60 min with an m.o.i. of ~ 10 :1 in 5 % CO₂ at 37 °C. The culture supernatants were removed and the infected cells were treated with DMEM containing gentamicin at 100 μ g ml⁻¹ for 60 min to kill extracellular bacteria. Triplicate wells for each strain were washed three times with PBS and the cell monolayers were lysed by adding 1 ml of ice-cold sterile, distilled water. The lysates were diluted tenfold for enumeration of viable bacteria on BHI agar plates, and these were considered as the 0 h numbers invading the cells. The remaining cells were subjected to further incubation for 6 h in 5 % CO₂ at 37 °C. Viable bacteria were enumerated as described above. Listerial survival or replication was expressed as fold-changes of counts at hour 6 relative to the 0 h numbers (mean \pm SD of three independent experiments, each performed in triplicate wells).

Statistical analysis. All data were analysed using the two-tailed Student’s *t*-test with *P* < 0.05 considered as statistically significant or *P* < 0.01 as of marked statistical significance.

RESULTS

ArcA of *L. monocytogenes* catalyses citrulline formation from arginine and shows increased transcription upon acid stress

The ArcA protein of *L. monocytogenes* has 30–52.2 % homology with ADIs of other micro-organisms (Fig. 1).

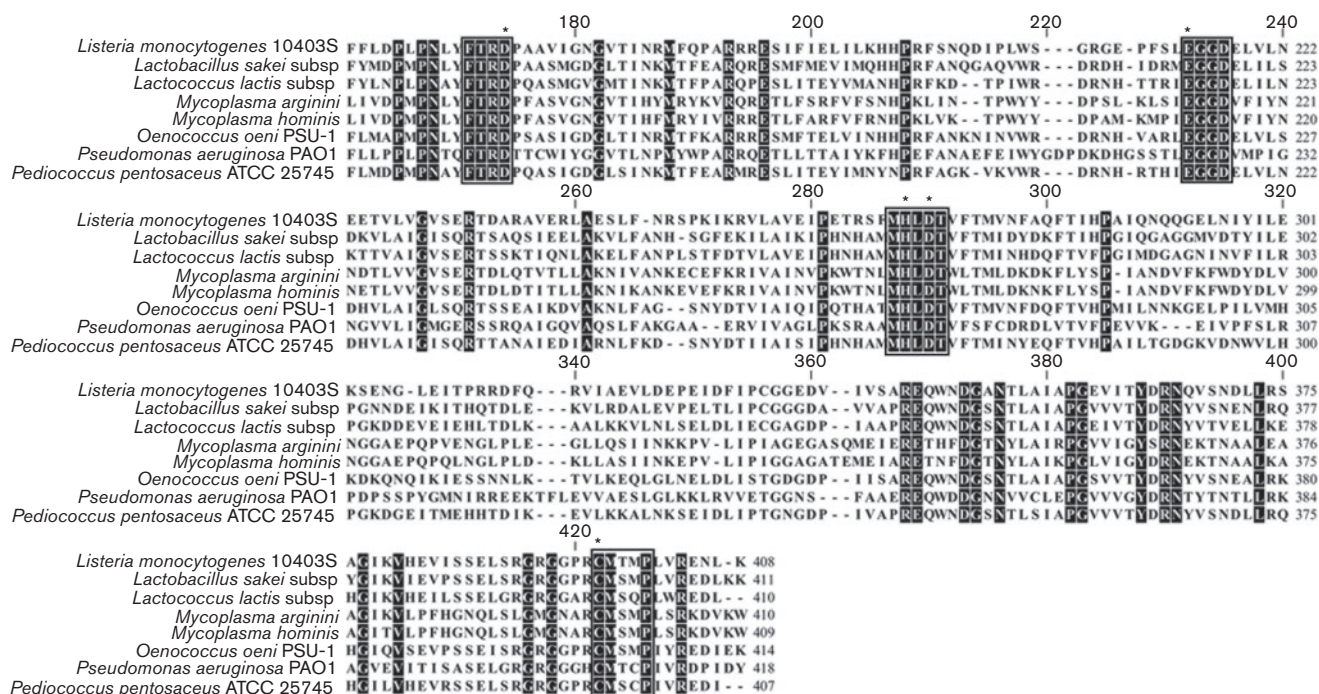


Fig. 1. Amino acid sequence analysis of ADIs from *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Mycoplasma arginini*, *Lactobacillus lactis* subsp. *lactis*, *Lactobacillus sakei* subsp. *sakei* 23K, *Oenococcus oeni* PSU-1, *Pediococcus pentosaceus* ATCC 25745 and *Mycoplasma hominis*. The highly conserved sequence motifs characteristic of ADI are boxed. The key amino acid residues with asterisks are involved in substrate binding and enzyme catalytic activity.

The ADI enzyme contains four conserved motifs (FTRD, EGGD, MHLDT and CMSxP) and a catalytic triad (Cys-His-Glu/Asp), which have been shown to play a significant role in substrate binding and catalytic activity (Galkin *et al.*, 2005; Li *et al.*, 2009; Lu *et al.*, 2006). We found that the putative motifs and the catalytic triad of ArcA are conserved in all 37 *L. monocytogenes* strains examined from the available database (Fig. 1), although the level of amino acid identity was at the lower end (93%), suggesting that it is an ADI homologue with arginine deimination activity. To extrapolate citrulline production by putative ADI of *L. monocytogenes*, we constructed a calibration curve that exhibited a good linear relationship between absorbance at 530 nm and citrulline concentrations ranging from 25 to 400 μM ($R^2=0.992$). Fig. 2(a) shows that citrulline production of the *arcA* deletion mutant (ΔarcA) was significantly lower than that of the wild-type strain (139.6 versus 187.2 μM , $P<0.01$), while the *arcA* complemented strain ($\text{C}\Delta\text{arcA}$) showed significantly higher enzyme activity than ΔarcA (267.8 versus 187.2 μM , $P<0.001$). We found that *arcA* mRNA transcription of wild-type and complemented strains was 5.4- and 9.2-fold higher, respectively, at pH 4.8 than at pH 7.0 ($P<0.01$ in both cases) (Fig. 2b). These results indicate that *arcA* encodes a functional ADI enzyme that might play a possible role in acid tolerance.

Deletion of *arcA* leads to decreased growth under acidic conditions and reduced survival in synthetic gastric fluid

The *arcA* mutant strain exhibited similar growth to its parent strain and the complemented strain both in BHI and in MEM media at neutral pH, suggesting that the *arcA* gene may not be essential for growth of *L. monocytogenes* under normal conditions (Fig. 3a, b). Deletion of *arcA* resulted in slower growth in BHI medium at pH 5.5 than the wild-type strain, with a significant difference starting from 7 h (OD₆₀₀ 0.283 versus 0.346, $P<0.01$) (Fig. 3a). The difference was more dramatic in MEM at pH 5.5, with a significant difference starting from 6 h (OD₆₀₀ 0.177 versus 0.248, $P<0.01$) (Fig. 3b). Such reduced growth was fully restored in the complemented strain $\text{C}\Delta\text{arcA}$ (Fig. 3a, b). To further determine whether *arcA* contributes to survival in the acidic stomach, *L. monocytogenes* wild-type, ΔarcA mutant and complemented strains were exposed to synthetic gastric fluid at pH 2.5. Fig. 3(c) indicates that the *arcA* deletion mutant exhibited significant reduction in survival, as compared with its parent (0.26 versus 2.65%, $P<0.05$) and complemented strains (0.26 versus 1.00%, $P<0.001$). These results suggest that *arcA* is involved in listerial resistance to acid stress.

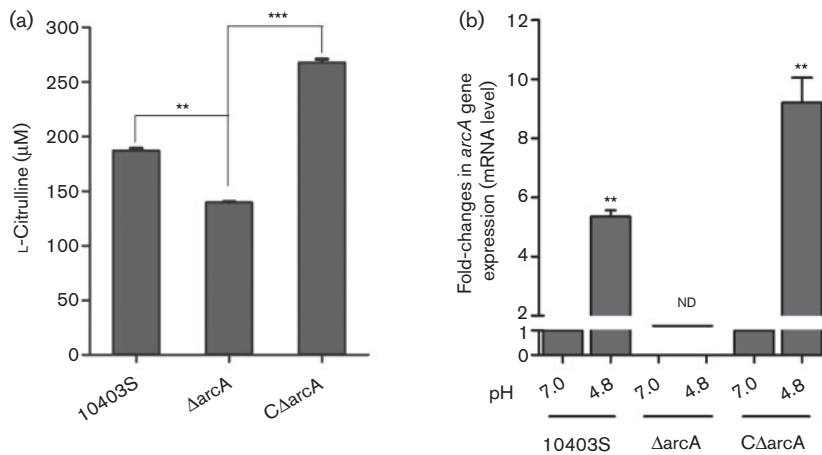


Fig. 2. Citrulline production by ADI of *L. monocytogenes*, *arcA* deletion mutant and its complemented strain (a) as well as *arcA* mRNA transcription of the wild-type strain 10403S, its mutant Δ*arcA* and complement strain CΔ*arcA* in BHI medium at pH 4.8 and 7.0 (b). Data are expressed as mean ± SD of three experiments. ** $P < 0.01$ and *** $P < 0.001$; ND, not detectable.

ArcA is involved in gastric survival in a mouse model

L. monocytogenes wild-type, *arcA* deletion mutant or complemented strains were intragastrically inoculated in mice to determine whether *arcA* is involved in survival in the gastrointestinal tracts. Fig. 4(a) shows that the *arcA* deletion mutant exhibited significantly lower survival in the stomach compared with that of the wild-type at 1 h (\log_{10} c.f.u. 2.9 versus 5.8, $P < 0.01$) or 2 h (\log_{10} c.f.u. 1.8 versus 4.0, $P < 0.01$) (Fig. 4a). Complementation of *arcA* restored its survival in the stomach to a level similar to the wild-type strain. These data indicate that the *arcA* gene plays an essential role in the survival of *L. monocytogenes* in the stomach, consistent with the observations obtained in the synthetic gastric model.

Deletion of *arcA* reduces virulence of *L. monocytogenes* in mice

The potential role of *arcA* in the virulence of *L. monocytogenes* was assessed *in vivo* using the ICR mouse model. Lethality was reduced by about fivefold in immunosuppressed mice intraperitoneally infected with the *arcA* deletion mutant, as compared with the wild-type strain (LD_{50} 1.1×10^5 versus 2.29×10^4 c.f.u.). In normal ICR mice, bacterial numbers recovered from spleens were significantly lower with the *arcA* deletion mutant than with the wild-type strain at 48 (\log_{10} c.f.u. 4.1 versus 6.7, $P < 0.001$) and 72 h (\log_{10} c.f.u. 3.8 versus 6.9, $P < 0.001$), and *arcA* complementation substantially improved its recovery (Fig. 4b), suggesting a role of *arcA* in *L. monocytogenes* virulence. ArcA might not play a part in listerial intracellular replication within macrophage cells as there were no significant differences in the fold-increase of the bacteria in RAW264.7 cells (representing intracellular replication) among the Δ*arcA* mutant and its parent strain ($P > 0.05$) (Fig. 4c).

DISCUSSION

L. monocytogenes has the ability to adapt to a wide range of environmental conditions (Begley *et al.*, 2010). Its tolerance

to low pH environments is of importance because the pathogen encounters acidic environments in nature and in the food processing industry, and *in vivo*, both during passage through the stomach and within the macrophage phagosome (O'Driscoll *et al.*, 1996). Therefore, it is important to understand, from a public health perspective, the mechanisms by which *L. monocytogenes* is able to tolerate such acidic conditions, which might in turn point to strategies for controlling this pathogen in foods. The low pH of the stomach is the first physical stress encountered by foodborne pathogens following ingestion (Begley *et al.*, 2005; Collins *et al.*, 2011; Cotter *et al.*, 2001b; Sleator *et al.*, 2007). Our previous study has revealed that ornithine transcarbamylase, encoded by *lmo0036* (an *arcB* paralogue), contributes to acid tolerance and virulence of *L. monocytogenes* by promoting the ADI and agmatine deiminase (AgDI) pathways (Chen *et al.*, 2011). The ADI system has been suggested as having a role in acid tolerance *in vitro* (Ryan *et al.*, 2009), which is additional to the GAD system (Cotter *et al.*, 2000) known to be involved in listerial acid resistance. It depends on ammonia production to protect bacteria from acid damage (Degnan *et al.*, 2000; Gruening *et al.*, 2006; Marquis *et al.*, 1987; Ryan *et al.*, 2009).

We clearly show, using gene knockout and genetic complementation together with mouse models, that the *arcA* orthologue in *L. monocytogenes* not only mediates acid tolerance *in vitro* but also participates in gastric survival and virulence in mice. Deletion of *arcA* led to reduced survival in synthetic stomach fluid as well as in the stomach of intragastrically inoculated mice and decreased recovery in the spleen of intraperitoneally inoculated mice. Ryan *et al.* (2009) found that *arcA* deletion resulted in decreased recovery of the mutant strain in the spleen, although genetic complementation was not tested. These findings are in general agreement with several other studies showing that mutant strains unable to mount an acid tolerance response display reduced virulence, compared with their parent strains, in mouse models (Chen *et al.*, 2011; Marron *et al.*, 1997), while acid-tolerant listeria strains are more invasive *in vitro* (Conte *et al.*, 2000) and more virulent *in vivo* (O'Driscoll *et al.*, 1996) than their acid-sensitive

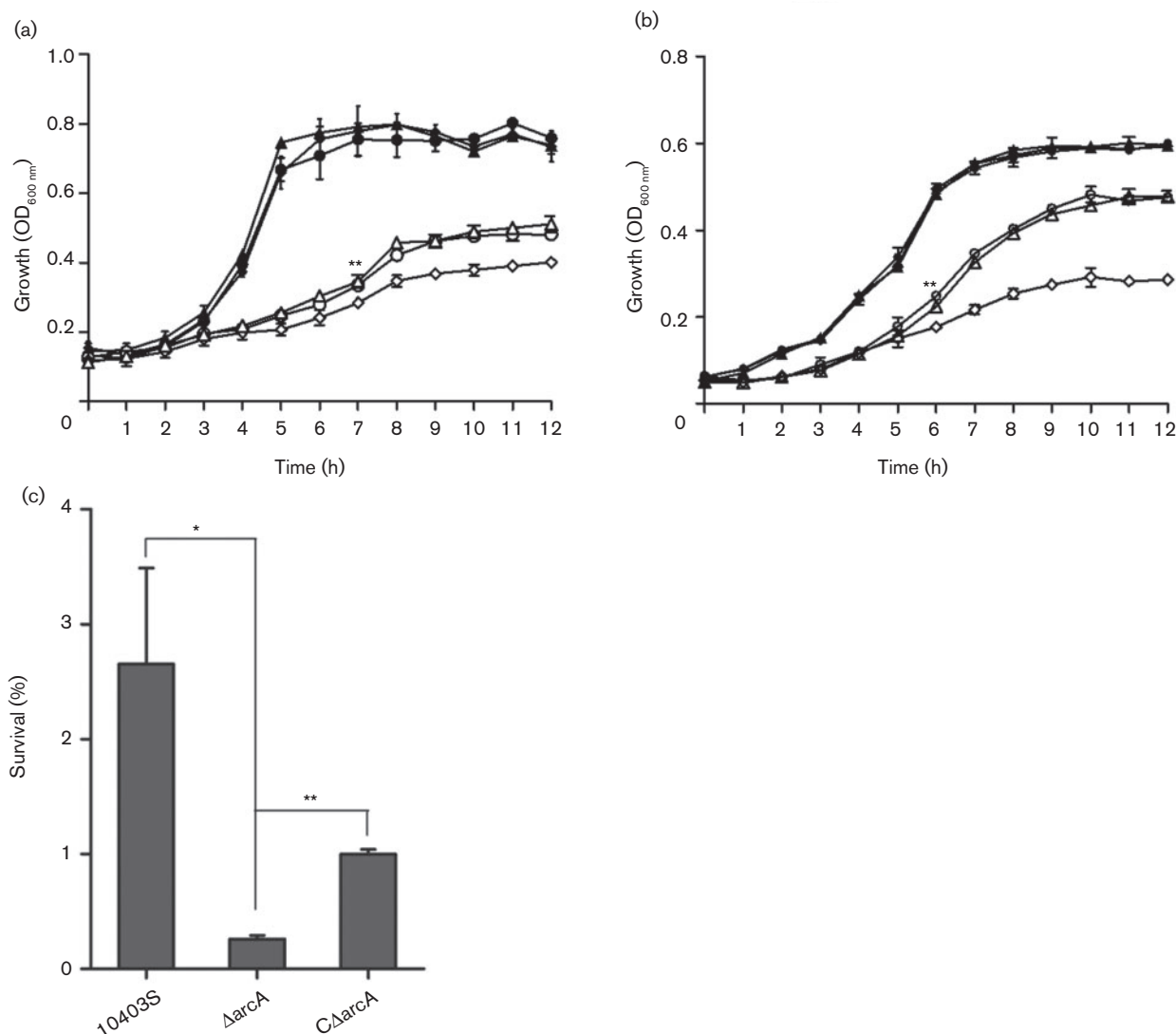


Fig. 3. Growth in BHI (a) and MEM media (b) at different pH (5.5 and 7.0) and survival in synthetic human gastric fluid at pH 2.5 (c) of *L. monocytogenes* wild-type 10403S, its mutant Δ arcA and complement strain C Δ arcA. Values represent mean \pm SD of triplicate wells in (a) and (b). ** $P < 0.01$ from hour points and thereafter between Δ arcA and its parent strain or C Δ arcA. In (a), pH 5.5: \circ , 10403S; \diamond , Δ arcA; \triangle , C Δ arcA; and pH 7.0: \bullet , 10403S; \blacklozenge , Δ arcA; \blacktriangle , C Δ arcA. In (b), pH 5.5: \square , 10403S; \diamond , Δ arcA; \triangle , C Δ arcA; and pH 7.0: \blacksquare , 10403S; \blacklozenge , Δ arcA; \blacktriangle , C Δ arcA. Data in (c) are expressed as mean \pm SD of three experiments. * $P < 0.05$, ** $P < 0.01$.

counterparts. The capacity to tolerate acid stress has been related to the virulence potential of other bacterial species. Degnan *et al.* (2000) found that a mutant lacking the *arcA* homologue gene had a survival disadvantage over wild-type *Streptococcus pyogenes* during initial stages of invasion. Therefore, we suggest that the ADI/AgDI pathways are involved in pathogenesis in a way parallel to that of the GAD system (Cotter *et al.*, 2005; Werbroutch *et al.*, 2009) by protecting *L. monocytogenes* from acid stress.

Decreased recovery of the Δ arcA mutant *in vivo* might be related to its susceptibility to the environments within the macrophages where phagosomes containing the engulfed

bacteria undergo gradual acidification to less than pH 5.5 (5.2–5.95, depending on the methods used for measurement) (Beauregard *et al.*, 1997; Birmingham *et al.*, 2008). However, we did not see marked changes in bacterial replication between the Δ arcA mutant and its parent strain or complemented strain in macrophage cells. This could be because *L. monocytogenes* is able to avoid the acidic environment by perforating the phagosomal vacuoles by means of listeriolysin O, expression of which is activated upon entering the cells (Schnupf & Portnoy, 2007). Therefore, the possible mechanism of reduced virulence *in vivo* attributable to Δ arcA deletion requires further experimental approaches.

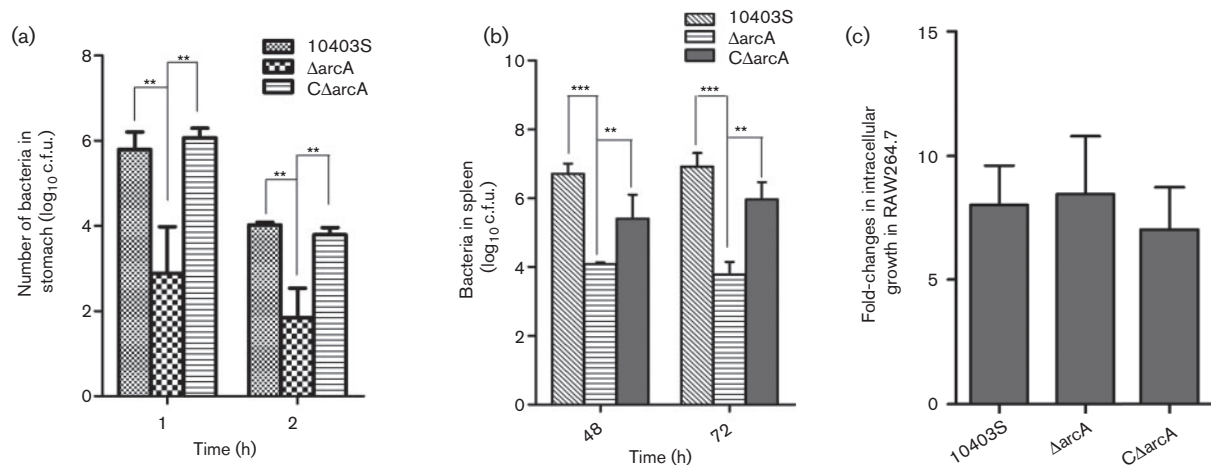


Fig. 4. Recovery of *L. monocytogenes* wild-type strain, its mutant $\Delta arcA$ and complemented strain $C\Delta arcA$ in the stomach from mice receiving intragastric inoculation (a), in spleen samples from mice with intraperitoneal inoculation (b) and in macrophages (c). Mice in (a) were inoculated with about $4\text{--}5 \times 10^8$ c.f.u. *Listeria* and stomachs were separated and homogenized for enumeration of *L. monocytogenes* at 1 and 2 h post-inoculation. Mice in (b) were inoculated with about $2\text{--}3 \times 10^5$ c.f.u. *Listeria* and spleen samples were separated at 48 and 72 h post-inoculation, and homogenized for bacterial enumeration. All data were expressed as mean \pm SEM of eight mice per group. ** $P < 0.01$ and *** $P < 0.001$. (c) Intracellular growth (expressed as fold-increase after 6 h incubation) of *L. monocytogenes* wild-type strain, its mutant $\Delta arcA$ and complemented strain in RAW264.7 macrophages.

We found that deletion of *arcA* significantly reduced citrulline production, but without complete abolishment (Fig. 2a). This is different from a previous report by Ryan *et al.* (2009) who showed that *arcA* deletion completely abolished citrulline production, shown as a colourless reaction mixture. The discrepancy could be due to the differences of colouring reagents used. We used the method of Knipp & Vasák (2000) in which thiosemicarbazide and Fe^{3+} were used to facilitate colour development, while these components were not used by Ryan *et al.* (2009). In *L. monocytogenes*, citrulline could be produced not only from arginine, but also from ornithine or *N*-acetylornithine in arginine biosynthesis by ornithine carbamoyltransferase (encoded by *lmo0036*, an orthologue of *arcB*) (Chen *et al.*, 2011). It could also come from glutamate biosynthesis by *N*-succinylornithine carbamoyltransferase encoded by *argF* (Lu *et al.*, 2006), which has its orthologue (*lmo1587*) in *L. monocytogenes*.

In conclusion, this study shows that ArcA initiates and promotes the ADI pathway and enables *L. monocytogenes* to overcome and tolerate acidic conditions in external environments and in the stomach. Additionally, ArcA might also contribute to the virulence of *L. monocytogenes*.

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