Lmo0036, an ornithine and putrescine carbamoyltransferase in *Listeria monocytogenes*, participates in arginine deiminase and agmatine deiminase pathways and mediates acid tolerance

Jianshun Chen,¹ Changyong Cheng,¹ Ye Xia,¹ Hanxin Zhao,¹ Chun Fang,¹ Ying Shan,¹ Beibei Wu² and Weihuan Fang¹

Correspondence

Weihuan Fang
whfang@zju.edu.cn

¹Zhejiang University Institute of Preventive Veterinary Medicine and Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine, 388 Yuhangtang Road, Hangzhou, Zhejiang 310058, PR China

²Zhejiang Provincial Center for Disease Control and Prevention, 630 Xincheng Road, Hangzhou, Zhejiang 310051, PR China

Listeria monocytogenes is a foodborne pathogen causing listeriosis. Acid is one of the stresses that foodborne pathogens encounter most frequently. The ability to survive and proliferate in acidic environments is a prerequisite for infection. However, there is limited knowledge about the molecular basis of adaptation of *L. monocytogenes* to acid. Arginine deiminase (ADI) and agmatine deiminase (AgDI) systems are implicated in bacterial tolerance to acidic environments. Homologues of ADI and AgDI systems have been found in *L. monocytogenes* lineages I and II strains. Sequence analysis indicated that *Lmo0036* encodes a putative carbamoyltransferase containing conserved motifs and residues important for substrate binding. *Lmo0036* acted as an ornithine carbamoyltransferase and putrescine carbamoyltransferase, representing the first example, to our knowledge, that catalyses reversible ornithine and putrescine carbamoyltransfer reactions. Catabolic ornithine and putrescine carbamoyltransfer reactions constitute the second step of ADI and AgDI pathways. However, the equilibrium of *in vitro* carbamoyltransfer reactions was overwhelmingly towards the anabolic direction, suggesting that catabolic carbamoyltransferase was probably the limiting step of the pathways. *Lmo0036* was induced at the transcriptional level when *L. monocytogenes* was subjected to low-pH stress. Its expression product in *Escherichia coli* exhibited higher catabolic carbamoyltransfer activities under acidic conditions. Consistently, absence of this enzyme impaired the growth of *Listeria* under mild acidic conditions (pH 4.8) and reduced its survival in synthetic human gastric fluid (pH 2.5), and corresponded to a loss in ammonia production, indicating that *Lmo0036* was responsible for acid tolerance at both sublethal and lethal pH levels. Furthermore, *Lmo0036* played a possible role in *Listeria* virulence.

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INTRODUCTION

Listeria monocytogenes is the causative agent of listeriosis, a severe disease that has an extremely high mortality rate (Freitag *et al.*, 2009; Liu, 2006). 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; Swaminathan & Gerner-Smidt, 2007). However, not all *L. monocytogenes* strains contribute equally to human

illness (Orsi *et al.*, 2011). Among the four evolutionary lineages, only lineages I and II are commonly isolated from contaminated foods and human listeriosis patients (Orsi *et al.*, 2011; Ward *et al.*, 2008). The virulence potential of *L. monocytogenes* is linked to its ability to survive adverse environmental conditions encountered both in natural environments and subsequently within the host (Ryan *et al.*, 2009; Sleator *et al.*, 2009). An elevated level of acid is often added to foods to inhibit bacterial growth (Byelashov *et al.*, 2010). After ingestion via contaminated foods, *L. monocytogenes* encounters the acidic conditions of the gastric barrier, followed by exposure to fatty acid in the intestine (Sleator *et al.*, 2009). Survival in the host gastrointestinal tract is the key stage for initiating listerial infection. Following cellular uptake, *L. monocytogenes* enters the acidic

Abbreviations: ADI, arginine deiminase; AgDI, agmatine deiminase; aOTC, anabolic OTC; aPTC, anabolic PTC; CK, carbamate kinase; cOTC, catabolic OTC; cPTC, catabolic PTC; OTC, ornithine carbamoyltransferase; PTC, putrescine carbamoyltransferase; qRT-PCR, quantitative RT-PCR; UPLC, ultraperformance liquid chromatography.

vacuole of the macrophage phagosome (de Chastellier & Berche, 1994). Undoubtedly, acid represents one of the most frequently encountered stresses for foodborne pathogens like *L. monocytogenes*. It should possess mechanisms for pH homeostasis that allow its survival and proliferation under acidic conditions.

The arginine deiminase (ADI) system has been characterized in several Gram-positive and Gram-negative bacteria and implicated in bacterial tolerance to acidic environments (Degnan *et al.*, 2000; Gruening *et al.*, 2006; Marquis *et al.*, 1987; Vrancken *et al.*, 2009a). Three enzymes constitute the deiminase pathway that catalyses the conversion of arginine into ornithine, NH_3 , CO_2 and ATP, namely ADI, catabolic ornithine carbamoyltransferase (cOTC) and carbamate kinase (CK), which are encoded by *arcA*, *arcB* and *arcC* respectively (Fig. 1a, c, e). Ornithine is transported out of the cell in exchange for a molecule of arginine by a transporter encoded by *arcD*. The byproduct NH_3 can combine with intracellular cytoplasmic protons to yield ammonium ions (NH_4^+), thereby increasing intracellular pH and protecting cells from acidic damage (Cunin *et al.*, 1986). In addition, ATP can be used to extrude protons via the F_0F_1 -ATPases, which play a role in pH homeostasis (Cotter *et al.*, 2000). A similar pathway has been described for agmatine, the decarboxylation product of arginine which yields putrescine

and ammonia upon catalysation (Griswold *et al.*, 2004; Llcer *et al.*, 2007; Lucas *et al.*, 2007). Less is known about the agmatine deiminase (AgDI) pathway, which involves the AgDI enzyme itself, catabolic putrescine carbamoyltransferase (cPTC) and CK (Fig. 1b, d, e) (Griswold *et al.*, 2004; Llcer *et al.*, 2007; Lucas *et al.*, 2007).

Homologues of ADI and AgDI systems have been found in the genomes of *L. monocytogenes* strains. To our knowledge, *L. monocytogenes* represents the first example harbouring both ADI and AgDI systems at the same genetic locus (Chen, 2010), e.g. the *lmo0036*–*lmo0043* region in the strain EGD-e (Glaser *et al.*, 2001). Interestingly, Joseph *et al.* (2006) detected the upregulation of *lmo0036* during infection of Caco-2 cells. Ryan *et al.* (2009) speculated that Lmo0036 was cOTC, subsequent to the functional characterization of ADI (encoded by *lmo0043*). However, Naumoff *et al.* (2004) suggested that Lmo0036 was a putrescine carbamoyltransferase (PTC) orthologue based on the systematic evolutionary tree of carbamoyltransferases from a variety of bacterial species including *L. monocytogenes*.

Functionally catabolic carbamoyltransferases have been found in a number of bacteria, e.g. *Enterococcus faecalis* (Llcer *et al.*, 2007), *Pseudomonas aeruginosa* (Sainz *et al.*, 1998), *Streptococcus mutans* (Casiano-Coln & Marquis, 1988) and *Lactobacillus hilgardii* (Arena *et al.*, 2002), and

Fig. 1. The ADI and AgDI pathways. OTC, ornithine carbamoyltransferase; PTC, putrescine carbamoyltransferase; CK, carbamate kinase.

these are of critical importance for ADI and AgDI pathways involved in pH homeostasis and microbial ecology. While cOTC mediates the decarbamylation of citrulline (the deimination product of arginine) yielding carbamoylphosphate and ornithine (Fig. 1c) (Legrain & Stalon, 1976), cPTC catalyses the decarbamylation reaction of carbamoylputrescine (the deimination product of agmatine) producing carbamoylphosphate and putrescine (Fig. 1d) (Wargnies *et al.*, 1979). However, little is known about the function of Lmo0036, the putative carbamoyltransferase in *L. monocytogenes*. Therefore, this study attempted to examine the functional activity of Lmo0036 and its roles in acid stress response and virulence potential of *L. monocytogenes*.

METHODS

Bacterial strains and growth conditions. *L. monocytogenes* reference strain 10403S was used as the wild-type strain and another 40 *L. monocytogenes* strains covering four lineages have been described in our previous studies (Chen *et al.*, 2009b, 2010a). *L. monocytogenes* and *Escherichia coli* strains were propagated at 37 °C in brain heart infusion (BHI) broth (Oxoid) and Luria–Bertani (LB) medium, respectively. Stock solutions of ampicillin (100 mg ml⁻¹), kanamycin (50 mg ml⁻¹) and chloramphenicol (50 mg ml⁻¹) were added to media, where appropriate, at the required levels.

Bioinformatic analysis. Amino acid sequences of Lmo0036 in *L. monocytogenes* and its homologues in other bacterial species were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). These sequences were aligned by CLUSTAL_X software (version 1.8). A phylogenetic tree was constructed using the Neighbor-Joining method of the Molecular Evolutionary Genetics Analysis software (MEGA version 4.0). The robustness of the branching pattern was tested by bootstrap analyses through 1000 replications. Putative enzyme active sites were predicted via the ExPASy Proteomics Server (<http://www.expasy.ch/>).

DNA manipulations. Genomic DNA was prepared using a protocol reported previously (Chen *et al.*, 2009c). Oligonucleotide primers

were synthesized by Invitrogen Biotechnology (Table 1). PCR was conducted using a PT200 thermal cycler (MJ Research), with the duration of extension depending on the expected length of the amplicon (1 min kb⁻¹, at 72 °C). PCR fragments were purified using the AxyPrep DNA Gel Extraction kit (Axygen) and digested with defined restriction enzymes (TaKaRa) to facilitate insertion into vectors. Positive clones were then sequenced by using the dideoxy method on an ABI-PRISM 377 DNA sequencer.

Construction of *lmo0036* knockout mutant. A homologous recombination strategy was used for in-frame deletion of the whole length of *lmo0036* (1026 bp) in *L. monocytogenes* 10403S according to a protocol described previously (Chen *et al.*, 2009d). Primer pairs 36-a/36-b and 36-c/36-d were used to generate fragments of 588 and 640 bp from flanking regions of *lmo0036* (Table 1). The two products were gel purified for splicing by overlap-extension PCR using external primer pair 36-a/36-d. The final 1216 bp product containing *Hind*III and *Eco*RI sites was digested and ligated to pUC18. After sequencing confirmation, the inserted fragment was subcloned into the thermo-sensitive shuttle vector pKSV7, which was then electro-transformed into the competent *L. monocytogenes* cells. Electrot transformants were grown at a non-permissive temperature (41 °C) in BHI containing chloramphenicol (10 µg ml⁻¹) to screen for chromosomal integration and the cointegrates were passaged successively in BHI without antibiotic at a permissive temperature (30 °C) to facilitate isolation of derivatives in which integrated plasmids had resolved by a second recombination event. The recombinants were identified as chloramphenicol-sensitive colonies and confirmed by PCR with primers 36-e/36-f (external to 36-a/36-d). The resultant knockout mutant was designated Δlmo0036.

Complementation of the *lmo0036* deletion. The *lmo0036* coding sequence (including the ribosome-binding site) was amplified from 10403S genomic DNA by PCR using primers 36-pER-F and 36-pER-R (Table 1). The 1278 bp product containing *Sac*I and *Bam*HI sites was digested and ligated to pUC18. After sequencing confirmation, the inserted fragment was subcloned into the vector pERL3. The resulting vector was electroporated into Δlmo0036 and a transformant was selected on BHI erythromycin plates. The resultant complemented mutant was designated Δlmo0036 + lmo0036.

Table 1. Oligonucleotide primers used in this study

Restriction sites incorporated into primer sequences are underlined.

Primer name	Primer (5'–3')	Amplicon size (bp)	Description
36-pET-F	CGAGGATCCATGAACAAAAAAGAGATT	1026	Used for cloning of <i>lmo0036</i>
36-pET-R	TATAAGCTTTTATAAGCCAGCGCCATTTC		
36-RT-F	CGGTCGCAAAATGAAAGAATC	145	Used for qRT-PCR
36-RT-R	CGCATGACCACCAAGCTGT		
gyrB-RT-F	AGACGCTATTGATGCCGATGA	91	
gyrB-RT-R	GTATTGCGCGTTGTCTTCGA		
36-a	ATAAGCTTTTATAAGCGTTTATGCAT	588	Used for construction of <i>lmo0036</i> null mutant
36-b	CCTTATTTTGGTATCTTCTATCACCTCA		
36-c	GATACCAAAATAAGGAGGAGAAGAGAGC	640	
36-d	TAGAATTCTCAAAAGTTGGAAGTAATGC		
36-c	GGATATGGTCCTACTGTTGGA	2392	Used to screen the positive clones of <i>lmo0036</i> null mutant
36-d	GCGTTGTTACCACTTCAAAAC		
36-pER-F	ATTGAGCTCGAGAGGCTAATAGACTGGAAA	1278	Used for complementation of the <i>lmo0036</i> deletion
36-pER-R	TATGGATCCTTATAAGCCAGCGCCATT		

Expression and purification of Lmo0036. The *lmo0036* gene was amplified by PCR using primers 36-pET-F and 36-pET-R (Table 1) and inserted into pET30a downstream of an in-frame sequence encoding an N-terminal histidine tag. The recombinant plasmid was transferred into *E. coli* Rosetta. *E. coli* cells carrying pET30a-*lmo0036* were grown until the cultures reached OD₆₀₀ 0.6; IPTG was added to a final concentration of 0.4 mM and the cultures were allowed to grow for an additional 3 h. The cells were collected, resuspended in PBS (50 mM, pH 7.2) and disrupted in a sonicator with 99 cycles of sonication at 300 W for 4 s with intermittent cooling on ice for 4 s, followed by centrifugation at 12 000 r.p.m. The supernatant was loaded onto a HiTrap chelating column saturated with a solution of NiCl₂. After washing the column with PBS containing 500 mM NaCl and 20 mM imidazole, bound protein was eluted with a linear gradient of 20–500 mM imidazole prepared in the same buffer. Protein concentration was determined on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). The preparations were analysed by SDS-PAGE, followed by Coomassie brilliant blue staining. *E. coli* cells carrying empty vector pET30a were employed as negative controls.

Site-directed mutagenesis. To identify the predicted active sites of Lmo0036, mutations (Table 2) were engineered into pET30a-*lmo0036* using the QuikChange Site-Directed Mutagenesis kit (Stratagene). All segments that were manipulated were sequenced to confirm the presence of the desired mutation and to exclude adventitious mutations. Expression and purification of variant Lmo0036 proteins were performed at optimal pH conditions as described above.

Determination of *in vitro* catabolic ornithine and putrescine carbamoyltransfer activities. The reaction was conducted at 37 °C and initiated by adding 0.5 µM purified Lmo0036 into solution A (8 mM citrulline or *N*-carbamoylputrescine, 240 mM arsenate and 8 mM uridine monophosphate, pH 2.0–9.0) and terminated at the indicated time points by adding 50 µl 50 % TCA. The enzyme activity was measured based on depletion of the substrates, citrulline or *N*-carbamoylputrescine, using a modified version of the method of Boyde & Rahmatullah (1980). The supernatant was mixed with a 1 ml aliquot of chromogenic solution (16.7 % sulphuric acid, 0.05 % butanedione monoxime, 0.01 % thiosemicarbazide). The samples were boiled for 5 min, cooled to room temperature in the dark and OD₅₃₀ was measured. One unit (U) of enzyme activity was defined as the amount of enzyme that was required for converting 1 µM substrate into 1 µM product min⁻¹ under the assay conditions. Citrulline or *N*-carbamoylputrescine standard was included as a positive control and the enzyme was added into solution A containing 50 µl 50 % TCA as a negative control.

Determination of *in vitro* anabolic ornithine and putrescine carbamoyltransfer activities. The reaction was initiated by adding 0.5 µM purified Lmo0036 into solution B (4 mM ornithine or putrescine, 4 mM carbamoylphosphate, 20 mM Tris/HCl, pH 4.0–11.0) and stopped at the indicated time points by adding 50 µl 50 %

TCA. This reaction was conducted at 37 °C. The enzyme activity was measured as shown above, based on the production of citrulline or *N*-carbamoylputrescine.

Analysis of biogenic amine by ultraperformance liquid chromatography (UPLC) combined with electrospray ionization tandem mass spectrometry (MS-MS). Analysis of biogenic amine (citrulline, ornithine, carbamoylputrescine or putrescine) in reaction samples was performed by UPLC-MS-MS with electrospray positive ionization (ESI+) as in previous studies (Ren *et al.*, 2007; Wu *et al.*, 2006). Briefly, an ACQUITY UPLC quaternary pump system equipped with the micro vacuum degasser, thermostat-equipped autosampler and thermostat-equipped column compartment (Waters) was coupled with a Micromass Quattro Ultima triple-quadrupole mass spectrometer. The analyte elution was carried out on a UPLC BEH C18 column (150 mm length, 2.0 mm internal diameter, 3.5 µm particle size; Waters) maintained at 25 °C with a run time of 3 min. The mobile phase was 10 % methanol:0.1 % formic acid in water with a flow rate of 0.2 ml min⁻¹. The conditions used for the electrospray source were as follows: capillary voltage, 3.5 kV; cone voltage, 50 V; source temperature, 100 °C; desolvation gas temperature, 350 °C; desolvation gas flow, 400 l nitrogen h⁻¹; cone gas flow, 45 l nitrogen h⁻¹; argon collision gas pressure to 3 × 10⁻³ mbar for MS-MS. The collision energy was optimized for each multiple reaction monitored transition.

Determination of ammonia in the culture supernatant. Production of ammonia in *L. monocytogenes* strain 10403S and its respective mutants, Almo0036 and Almo0036 + lmo0036, was measured by using the Ammonia Assay kit (Sigma) using a modified version of the method of Fulde *et al.* (2011). For this, bacteria were grown in trypticase soya broth with yeast extract (TSBY) medium and harvested by centrifugation 24 h after inoculation. The amount of ammonia (mg ml⁻¹) in the supernatant was determined according to the manufacturer's instructions. The ammonia production in the parent strain was set at 100 %.

Quantitative reverse transcriptase PCR. *L. monocytogenes* strain 10403S was grown in BHI to stationary phase (OD₆₀₀ 0.6). The culture was divided into two portions: one was exposed to sublethal acidic conditions (pH 4.8) and the other to neutral conditions (pH 7.0). After 60 min exposure, bacterial cells were collected by centrifugation and subjected to total RNA extraction using a Trizol extraction kit according to the manufacturer's instructions. DNase I (Fermentas) digestion was performed to break down contaminating genomic DNA. The resulting DNA-free RNA was converted to cDNA using Reverse Transcriptase (Promega). The concentration and purity of cDNA were evaluated by using a Nanodrop ND-1000 spectrophotometer.

The quantitative real-time PCR (qRT-PCR) was performed using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories) and a SYBR Premix Ex *Taq* (perfect real-time) PCR kit (TaKaRa) with primers listed in Table 1. *lmo0036* was cloned into pMD18-T and a 10-fold dilution series of the recombinant plasmid was used to

Table 2. Mutations in active sites of Lmo0036 by site-directed mutagenesis

Amino acid position	Putative function	Substitution	Primer
54–58	Carbamoyl phosphate binding	STRTR→AAAAA	TTGAACAATCAGCAGCAGCAGCAGCAGTATCTTTTGAACACGCA TGCTGTTTCAAAAGATACTGCTGCTGCTGCTGCTGATTGTTCAA
105	Carbamoyl phosphate binding	R→A	TGAAGTTTATGGCCGAGCAGCCGCAACTCGTGGGGAA TTCCCCACGAGTTGCGGCTGCTGCGGCCATAAATTCA
271–274	Putrescine/ornithine binding	HCLP→AAAA	ATATTGATGGCGGCAGTTGAACGTCATCAA TTGATGACGTTCAACTGCCGCCATCAATAT

construct a standard curve for qRT-PCR. The transcriptional levels of *lmo0036* were calculated based on the standard curve. The housekeeping gene *gyrB* was selected as the reference gene for normalization (Table 1). RNA was extracted from three separate cultures and all qRT-PCRs were performed in triplicate (E value >0.99). The transcription level of *lmo0036* at pH 7.0 was set as 100 %.

Growth in sublethal acidic conditions. *L. monocytogenes* strains 10403S and Δ *lmo0036* were cultured to stationary phase (OD₆₀₀ 0.6), washed in PBS (0.01 M, pH 7.2) and 1:100 diluted in BHI adjusted to pH 4.8. Samples (200 μ l) were removed and added to individual wells of a 96-well plate. Growth at 37 °C was measured at OD₆₀₀ for 12 h. This experiment was repeated three times, in triplicate wells for each strain.

To further determine the role of *Lmo0036* in listerial growth under acidic conditions, a competitive growth experiment was performed as previously reported (Milillo *et al.*, 2009). Briefly, stationary phase cultures of 10403S and Δ *lmo0036* were mixed in a 1:1 ratio, followed by 1:100 dilution into BHI at pH 4.8. A 10-fold dilution series of the mixed cultures was plated onto BHI plates at two time points corresponding to mid-exponential and stationary phases (i.e. 6 and 12 h after inoculation). At each time point, 40 colonies were subjected to PCR analysis to identify colonies as the mutant or parent strain using primers 36-a/36-d (Table 1). This assay was run in triplicate three times.

Survival in synthetic gastric fluid. Stationary phase cultures of *L. monocytogenes* 10403S and its mutant Δ *lmo0036* were harvested, washed in PBS and resuspended in synthetic human gastric fluid (8.3 g proteose peptone, 3.5 g D-glucose, 2.05 g NaCl, 0.6 g KH₂PO₄, 0.11 g CaCl₂, 0.37 g KCl, 0.05 g bile, 0.1 g lysozyme and 13.3 mg pepsin, all l⁻¹; pH 2.5). Changes in the cell population were determined by plate counting after 30 min of incubation. The test was run in triplicate three times.

Mouse virulence assays. The virulence of Δ *lmo0036* and its parent strain 10403S was assessed using ICR mice (female, 20 g) as described previously (Jiang *et al.*, 2011). The animal experiment was approved by the Laboratory Animal Management Committee of Zhejiang University and mice were handled under strict ethical conditions. Briefly, five groups (six per group) of mice were inoculated intraperitoneally with 0.2 ml aliquots of the appropriately diluted *Listeria* strain resuspended in PBS. Mice in the control group received injections of PBS. The group inoculated with appropriately 5×10^5 bacterial cells were killed 3 days post-infection. Spleens were homogenized in PBS (0.01 M, pH 7.2) and surviving cells enumerated by plating serial dilutions of the homogenized organs on BHI agar. The LD₅₀ values were calculated by using the trimmed Spearman–Kärber method on the basis of mouse mortality data recorded during a 10 day post-injection period.

Statistical analysis. The two-tailed Student's *t*-test was used for comparison of data, where necessary, and *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Lmo0036 is conserved in *L. monocytogenes* lineages I and II

Lmo0036 was highly conserved in *L. monocytogenes* lineages I and II strains with 99 % sequence identity at the amino acid level. *Lmo0036* showed higher similarity to its orthologues in other bacteria, including members of the genera *Streptococcus*, *Enterococcus*, *Pediococcus*, *Lactobacillus* and *Lactococcus* (64.6–74.5 %), than to those in *Mycoplasma* (49.6–51.0 %) (Fig. 2a). *Lmo0036* and its orthologues

shared the same conserved motif and residues, including sites for substrate binding and for structural integrity (Fig. 2b), suggesting that *Lmo0036* is a typical carbamoyltransferase containing intact functional residues.

Expression and purification of *Lmo0036*

To analyse the enzyme activity of *Lmo0036*, *Lmo0036* was overexpressed in *E. coli* Rosetta following IPTG induction. The majority of the expression product was in soluble form and only a small portion was formed as inclusion bodies (Fig. 3a). His-tagged recombinant *Lmo0036* was purified to homogeneity by nickel-chelated affinity column chromatography. Its purity was confirmed by SDS-PAGE as indicated by a single band at the expected molecular mass (Fig. 3b).

Lmo0036 catalyses reversible *in vitro* ornithine carbamoyltransfer reactions

The *in vitro* catabolic and anabolic ornithine carbamoyltransfer activities of *Lmo0036* were tested using citrulline and ornithine as substrates respectively. The ornithine carbamoyltransfer activities varied at different pHs, the catabolic direction peaked under acidic conditions with an optimal pH of 5.0–6.0 (Fig. 4a) and the anabolic direction was highest under alkaline conditions with an optimal pH of 10.0 (Fig. 4b). *Lmo0036* exhibited significantly higher specific activity of anabolic ornithine carbamoyltransferase (aOTC) (3.59 U mg⁻¹) than that of cOTC (0.12 U mg⁻¹) (Table 3). The reaction products, ornithine or citrulline, were confirmed by UPLC-MS-MS analysis (data not shown). These results indicate that *Lmo0036* acts as an OTC catalysing reversible ornithine carbamoyltransfer reactions.

Less than 18 % of the aOTC activity of variant *Lmo0036* proteins, harbouring mutations in positions 54–58 (STRTR→AAAAA), 105 (R→A) and 271–274 (HCLP→AAAA) (Table 2), were retained at pH 5.0 ($P < 0.01$) (Fig. 5a), demonstrating that these sites were crucial for the ornithine carbamoyltransferase (OTC) activity.

Lmo0036 mediates reversible *in vitro* putrescine carbamoyltransfer reactions

The *in vitro* catabolic and anabolic putrescine carbamoyltransfer (aPTC) activities of *Lmo0036* were determined using carbamoylputrescine and putrescine as substrates, respectively. Putrescine carbamoyltransfer activities varied with pH, with catabolic activity being highest under acidic condition with an optimal pH of 5.0 (Fig. 4c) and anabolic activity highest under alkaline condition at pH 8.0 (Fig. 4d). This enzyme showed significantly higher specific activity of aPTC (35.60 U mg⁻¹) than that of cPTC (0.57 U mg⁻¹) (Table 3). The reaction products, putrescine or carbamoylputrescine, were confirmed by HPLC-MS-MS analysis (data not shown). These data illustrate that *Lmo0036* also acts as a putrescine carbamoyltransferase (PTC) catalysing reversible putrescine carbamoyltransfer reactions.

Fig. 2. (a) Phylogenetic tree of Lmo0036 and its homologues from diverse bacterial species. The values above and below the horizontal lines (expressed as percentages) indicate the robustness of the corresponding branches, as determined by a bootstrap analysis evaluated from 1000 replications. (b) Conserved motifs and residues among bacterial species are boxed. Residues required for carbamoylphosphate (*) and putrescine/ornithine (■) binding and for structural integrity (◆) are indicated above the alignment.

On the other hand, the aPTC activity of variant Lmo0036 proteins, containing mutations in positions 54–58 (STRTR→AAAAA), 105 (R→A) and 271 to 274 (HCLP→AAAA) (Table 2), decreased to significantly lower levels when incubated at pH 5.0 ($P < 0.01$) (Fig. 5b), indicating that these sites were also critical for the PTC reactivity.

Deletion of *lmo0036* leads to a loss in ammonia production

Since Lmo0036 is speculated to be a carbamoyltransferase participating in ADI and AgDI pathways which play a role in acid tolerance via ammonia generated in the first and

third steps (Fig. 1a, b, e), production of ammonia was monitored in *L. monocytogenes* 10403S and its mutants Δ lmo0036 and Δ lmo0036 + lmo0036. Ammonia production displayed a 40 % decrease in Δ lmo0036 compared with its parent strain and complemented strain ($P < 0.01$), indicating an important role of Lmo0036 in bacterial ammonia production.

Transcriptional analysis of *lmo0036* under acidic conditions

As the ADI or AgDI pathway confers a possible contribution to bacterial acid tolerance, alterations in expression of

Fig. 3. SDS-PAGE analysis of the recombinant Lmo0036 protein. (a) Expression of Lmo0036. Lanes: M, molecular mass standards; 1, inclusion body of *E. coli* Rosetta harbouring pET30a; 2, supernatant of *E. coli* Rosetta harbouring pET30a; 3, inclusion body of *E. coli* Rosetta harbouring pET30a-Lmo0036; 4, supernatant of *E. coli* Rosetta harbouring pET30a-Lmo0036. (b) Purification of Lmo0036. Lanes: M, molecular mass standards; 1, *E. coli* Rosetta harbouring pET30a-Lmo0036.

lmo0036 of *L. monocytogenes* 10403S under acidic conditions were examined by qRT-PCR. Expression of *lmo0036* showed around a fourfold increase under acidic conditions (pH 4.8), compared with that under neutral conditions (pH 7.0) ($P<0.01$), suggesting a possible role of the *lmo0036* gene product in acid adaptation.

Δ lmo0036 exhibits slower growth under sublethal acidic conditions

The mutant Δ lmo0036 exhibited a similar growth rate to its parent strain in BHI at neutral pH 7.0 (data not shown), indicating that *lmo0036* was not essential for listerial growth under normal conditions. Exposure to sublethal acidic conditions (pH 4.8) resulted in a significant reduction ($P<0.05$) in growth rate and in final optical densities of Δ lmo0036 relative to its parent strain and complemented strain (Fig. 6a).

To more sensitively determine the role of Lmo0036 in acid tolerance under sublethal acidic conditions, competitive growth experiments were performed in BHI adjusted to pH 4.8. Significantly lower numbers of Δ lmo0036 were recovered at both the mid-exponential and early stationary phase time points ($P<0.05$) (Fig. 6b), indicating that Lmo0036 contributed to enhanced acid tolerance under mildly (sublethal) acidic conditions.

Fig. 4. Relative enzymic activities of purified Lmo0036 under different pHs measured after 60 min of incubation. (a) Catabolic ornithine carbamoyltransfer activity. (b) Anabolic ornithine carbamoyltransfer activity. (c) Catabolic putrescine carbamoyltransfer activity. (d) Anabolic putrescine carbamoyltransfer activity. The peak values of the four carbamoyltransfer activities were set at 100%.

Table 3. Optimal pH and specific activity (U mg⁻¹) of Lmo0036 in *L. monocytogenes*

	Direction	Optimal pH	Specific activity (U mg ⁻¹)
Ornithine carbamoyltransferase (OTC)	Catabolic	5.0–6.0	0.12
	Anabolic	10.0	3.59
Putrescine carbamoyltransferase (PTC)	Catabolic	5.0	0.57
	Anabolic	8.0	35.60

Δlmo0036 shows decreased survival ability in lethal acidic conditions

To clarify the contribution of Lmo0036 to the survival of *L. monocytogenes* at lethal pH, survival was examined in synthetic human gastric fluid adjusted to pH 2.5. The Δlmo0036 mutant exhibited a 10-fold reduction in the survival rate compared with the parent strain and complemented strain ($P < 0.01$) (Fig. 6c), suggesting that Lmo0036 is involved in the acid resistance of *L. monocytogenes* under potentially lethal pH conditions.

Fig. 5. Relative enzyme activities of wild-type and recombinant variant Lmo0036 proteins measured at the indicated time points. (a) Anabolic ornithine carbamoyltransfer reactivity. (b) Anabolic putrescine carbamoyltransfer reactivity. The peak values of carbamoyltransfer activities of wild-type Lmo0036 were set at 100 %. (a, b) ◆, Lmo0036; ■, Lmo0036-R105A; ▲, Lmo0036-STRTR54~58AAAAA; □, Lmo0036-HCLP271~247AAAA.

Lmo0036 is implicated in the virulence of *L. monocytogenes*

To examine whether the reduced acid tolerance of *L. monocytogenes* results in impaired bacterial *in vivo* virulence, we calculated the LD₅₀ values and enumerated the surviving cells in the spleens 3 days post-infection in an intraperitoneally inoculated murine model. LD₅₀ of Δlmo0036 (LD₅₀~6.2 × 10⁵ c.f.u.) was found to be higher than that of its parent strain (LD₅₀~8.5 × 10⁴ c.f.u.) and complemented strain (LD₅₀~7.8 × 10⁴ c.f.u.). Also, Δlmo0036 exhibited an approximately 16-fold decrease in survival in the spleen relative to its parent strain and an 11-fold decrease relative to the complemented strain ($P < 0.05$) (Fig. 6d), indicating that deletion of Lmo0036 influences the ability of *L. monocytogenes* to cause invasive disease in mice.

DISCUSSION

Acid represents one of the most frequently encountered stresses for foodborne pathogens, including *L. monocytogenes* (Bowman *et al.*, 2010; Cotter & Hill, 2003). The ability to survive and grow to high numbers in acidic environments is critical for *L. monocytogenes* to initiate systemic infection (Sleator *et al.*, 2009). Thus, it is important to understand the mechanisms which *L. monocytogenes* utilizes to overcome this hurdle, which might in turn point to strategies for controlling this pathogen in foods. The glutamate decarboxylase (GAD) system plays a role in *L. monocytogenes* survival at low pH (Cotter *et al.*, 2001) and the ADI and AgDI systems encoded by *lmo0036*–*lmo0043* may also contribute to stabilizing the cytoplasmic pH in *L. monocytogenes* (Chen *et al.*, 2009d; Ryan *et al.*, 2009). Ryan *et al.* (2009) identified that functional ADI (encoded by *lmo0043*) has a role in initiating the ADI pathway. Chen *et al.* (2009d) also determined the role of AgDI (encoded by *lmo0038*) in acid stress response, which triggers the putative AgDI pathway. It is tempting to speculate that functional ADI and AgDI systems work in parallel in *L. monocytogenes*.

Lmo0036 represents the first example of a carbamoyltransferase which is able to catalyse reversible ornithine and putrescine carbamoyltransfer reactions

OTC is widely distributed in Gram-positive and Gram-negative bacteria and comprises two subtypes, aOTC and cOTC (Arena *et al.*, 2002; Casiano-Colón & Marquis, 1988; Legrain & Stalon, 1976; Sainz *et al.*, 1998). aOTC catalyses the carbamoylation of ornithine to form citrulline participating in *de novo* biosynthesis of arginine, while cOTC performs the decarbamoylation of citrulline to form ornithine and carbamoylphosphate, which represents the second step of the ADI pathway (Cunin *et al.*, 1986; Legrain & Stalon, 1976) (Fig. 1c). Members of the genera *Pseudomonas*, *Aeromonas*, *Proteus* and *Enterococcus* possess two distinct enzymes, aOTC (encoded by *argF*) and cOTC (encoded by *arcB*), catalysing the anabolic or catabolic

Fig. 6. Growth and survival of *L. monocytogenes* 10403S (◆) and its mutant strains Δ lmo0036 (◇) and Δ lmo0036+lmo0036 (■) under acidic conditions *in vitro* and in mice. (a) Growth of the *L. monocytogenes* strains in brain heart infusion (BHI) under sublethal acidic conditions (pH 4.8). (b) Competitive growth of the parent strain (white) and Δ lmo0036 (black) in BHI under sublethal acidic conditions (pH 4.8) as shown by respective percentages in the mixture. (c) Survival of the *L. monocytogenes* strains in synthetic human gastric fluid (pH 2.5). (d) Comparison of virulence of the *L. monocytogenes* strains in ICR mice. Bars indicate the numbers of *L. monocytogenes* cells recovered from the spleens 3 days post-infection. Error bars indicate SD. * $P < 0.05$; ** $P < 0.01$.

reaction, respectively (De la Fuente *et al.*, 1996). *E. coli* K-12 harbours two genes, *argF* and *argI*, encoding two homologues of aOTC (Cunin *et al.*, 1986). Notably, aOTC is also able to catalyse the cOTC reaction in the majority of bacterial genera except *Pseudomonas*, whilst cOTC fails to mediate and, in some cases, even represses *in vivo* anabolic activity (Cunin *et al.*, 1986; Sainz *et al.*, 1998).

Relatively less is known about PTC. This enzyme has only been identified in a small number of bacteria (Llácer *et al.*, 2007; Lucas *et al.*, 2007). PTC and OTC both belong to the aspartate carbamoyltransferase/OTC (ATC/OTC) family. At least two differences are found between these two carbamoyltransferases. First, PTC possesses both anabolic and catabolic activities in most cases (Wargnies *et al.*, 1979) (Fig. 1d). The catabolic reaction represents the second step of the AgDI pathway (Lucas *et al.*, 2007). Second, in contrast with OTC being substrate-specific, PTC utilizes a wide range of substrates other than putrescine, including ornithine and several diamines (diaminopropane, diaminohexane, spermine, spermidine, cadaverine). However, specific activities of PTC using these alternative substrates are apparently lower (Wargnies *et al.*, 1979).

Determination of the carbamoyltransferase activities usually relies on isotopic tracing and NMR methods (Legrain & Stalon, 1976; Sainz *et al.*, 1998). These approaches are complicated, time-consuming and less visual. Thus, we used biochemical-coloration reactions combined with UPLC-MS-MS analysis to provide convincing evidence for the enzymic activities of Lmo0036. Generation of ornithine or putrescine is determined based on the production of a red colour (catabolic), while consumption of citrulline or carbamoylputrescine is linked to the colour turning lighter pink (anabolic). Kinetic studies revealed that the equilibrium of *in vitro* ornithine or putrescine carbamoyltransfer

reactions lies overwhelmingly towards the formation of citrulline or carbamoyl (anabolic) (Legrain & Stalon, 1976; Legrain *et al.*, 1977; Sainz *et al.*, 1998).

The *lmo0036*–*lmo0043* cluster in *L. monocytogenes* represents the first example, to our knowledge, of ADI and AgDI systems at the same genetic locus (Chen, 2010). *lmo0036* is the first gene in this cluster upstream of *lmo0043* (ADI) and *lmo0038* (AgDI), and encodes the only putative carbamoyltransferase therein (Fig. 2). Lmo0036 contains the conserved motif and residues, including sites for substrate binding and for structural integrity (Fig. 2b; Fig. 5), suggesting that Lmo0036 is a typical carbamoyltransferase. This enzyme represents the first example, to our knowledge, of carbamoyltransferase catalysing reversible ornithine and putrescine carbamoyltransfer reactions (Fig. 4). It exhibited higher *in vitro* specific activities of aOTC and aPTC than those of cOTC and cPTC (Table 3), consistent with previous reports that *in vitro* OTC or PTC reactions lie towards the anabolic direction (Legrain & Stalon, 1976; Legrain *et al.*, 1977; Sainz *et al.*, 1998).

Lmo0036 promotes ADI and AgDI pathways and mediates acid tolerance

ADI deiminates the substrate arginine producing citrulline and NH_3 (Fig. 1a). Citrulline is phosphorylated from phosphate (Pi) by cOTC, yielding carbamoylphosphate and ornithine (Fig. 1c) (Cunin *et al.*, 1986; Vrancken *et al.*, 2009b). On the other hand, AgDI initiates its pathway via deimination of agmatine generating carbamoylputrescine and NH_3 (Fig. 1b). Carbamoylputrescine is phosphorylated from phosphate by cPTC producing carbamoylphosphate and putrescine (Fig. 1d) (Landete *et al.*, 2008; Llácer *et al.*, 2007). The final reactions of both ADI and AgDI pathways require CK with the end products ATP, CO_2 and NH_3 from

carbamoylphosphate (Fig. 1e) (Ramón-Maiques *et al.*, 2010). For each mole of arginine or agmatine catabolized via the deiminase pathway, two moles of ammonia are produced. The ammonia can combine with intracellular cytoplasmic protons to yield ammonium ions (NH_4^+), thereby alleviating acidification of the cytoplasm and contributing to pH homeostasis (Cunin *et al.*, 1986). Thus, ADI and AgDI pathways have been proposed to be important for acid stress response. ADI or AgDI, catabolic carbamoyltransferase and CK represent three important enzymes responsible for the pathways. As ADI or AgDI catalyses an essentially irreversible reaction and, also, as the reaction mediated by CK is significantly in favour of ATP synthesis, catabolic carbamoyltransferase is probably the limiting step of the pathway (Legrain & Stalon, 1976; Legrain *et al.*, 1977). Kinetic studies supported this hypothesis; we observed that specific activities of cOTC and cPTC are considerably lower than those of aOTC and aPTC (Table 3).

The gene *lmo0036* is induced at the transcriptional level under low pH and its product Lmo0036 exhibits significantly higher cOTC and cPTC activities under acidic conditions (Fig. 4a, c; Table 3), suggesting that Lmo0036 is a critical enzyme that is required for the acid tolerance systems. Subsequently, we examined the role of Lmo0036 using an *lmo0036* knockout mutant and its complemented mutant. Absence of this enzyme impairs the growth of *L. monocytogenes* in BHI under mild acidic condition (pH 4.8) (Fig. 6a, b) and also attenuates the survival ability in synthetic human gastric fluid (pH 2.5) (Fig. 6c), which corresponds to a decrease in ammonia production ($P < 0.01$). These results consistently illustrate that deletion of Lmo0036 inactivates the ADI and AgDI pathways, which are required for maximal acid tolerance at both sublethal and lethal pH levels. The first step (a deimination reaction producing 1 mol ammonia) of the ADI and AgDI pathways mediated by ADI and AgDI is apparently functional in Δ lmo0036. However, accumulation of the product, citrulline or carbamoylputrescine, might affect the reaction equilibrium, thereby leading to a decreased efficiency of the deimination reaction and reduced generation of ammonia.

The ability to tolerate or resist acid stress has been implicated in the virulence potential of a number of bacteria (Ryan *et al.*, 2009; Sainz *et al.*, 1998). The acid-tolerant *L. monocytogenes* strains are more invasive *in vitro* (Conte *et al.*, 2000) and more virulent *in vivo* (O'Driscoll *et al.*, 1996) than their acid-sensitive counterparts, and the reverse has also been established. Consistently, a mutant unable to induce acid tolerance response displays subdued virulence compared with its parent strain in mice (Marron *et al.*, 1997). This is also the case for Δ lmo0036, although absence of Lmo0036 did not affect the *in vitro* invasive ability in HeLa epithelial cells (data not shown), but impaired the *in vivo* virulence in mice (Fig. 6d). In addition, *lmo0036* is conserved in *L. monocytogenes* lineages I and II and another pathogenic species *Listeria ivanovii*, but is absent from non-pathogenic species (*Listeria*

innocua, *Listeria welshimeri* and *Listeria seeligeri*) and also specifically absent from *L. monocytogenes* lineages III and IV, which appear to be rarely associated with human morbidity (Chen *et al.*, 2009c, 2010b; Orsi *et al.*, 2011; Ward *et al.*, 2008), further suggesting that this enzyme is involved in the pathogenesis of *L. monocytogenes*, most likely by its acid tolerance mechanisms.

In conclusion, *L. monocytogenes* harbours a functional Lmo0036 catalysing reversible ornithine and putrescine carbamoyltransfer reactions. This enzyme promotes ADI and AgDI pathways that contribute to the acid tolerance and virulence of the bacterium. Lmo0036 represents, to our knowledge, the first enzyme involved in the ADI and AgDI pathways in *L. monocytogenes* to be reported.

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