Arginine deiminase pathway is far more important than urease for acid resistance and intracellular survival in Laribacter hongkongensis: a possible result of arc gene cassette duplication

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Abstract

Background: Laribacter hongkongensis is a Gram-negative, urease-positive bacillus associated with invasive bacteremic infections in liver cirrhosis patients and fish-borne community-acquired gastroenteritis and traveler’s diarrhea. Its mechanisms of adaptation to various environmental niches and host defense evasion are largely unknown. During the process of analyzing the L. hongkongensis genome, a complete urease cassette and two adjacent arc gene cassettes were found. We hypothesize that the urease cassette and/or the arc gene cassettes are important for L. hongkongensis to survive in acidic environment and macrophages. In this study, we tested this hypothesis by constructing single, double and triple non-polar deletion mutants of the urease and two arc gene cassettes of L. hongkongensis using the conjugation-mediated gene deletion system and examining their effects in acidic environment in vitro, in macrophages and in a mouse model.

Results: HLHK9ΔureA, HLHK9ΔureC, HLHK9ΔureD and HLHK9ΔureE all exhibited no urease activity. HLHK9ΔarcA1 and HLHK9ΔarcA2 both exhibited arginine deiminase (ADI) activities, but HLHK9ΔarcA1/arcA2 double deletion mutant exhibited no ADI activity. At pH 2 and 3, survival of HLHK9ΔarcA1/arcA2 and HLHK9ΔureA/arcA1/arcA2 were markedly decreased (p < 0.001) but that of HLHK9ΔureA was slightly decreased (p < 0.05), compared to wild type L. hongkongensis HLHK9. Survival of HLHK9ΔureA/arcA1/arcA2 and HLHK9ΔarcA1/arcA2 in macrophages were also markedly decreased (p < 0.01 and p < 0.01 respectively) but that of HLHK9ΔureA was slightly decreased (p < 0.05), compared to HLHK9, although expression of arcA1, arcA2 and ureA genes were all upregulated. Using a mouse model, HLHK9ΔureA exhibited similar survival compared to HLHK9 after passing through the murine stomach, but survival of HLHK9ΔarcA1/arcA2 and HLHK9ΔureA/arcA1/arcA2 were markedly reduced (p < 0.01).

Conclusions: In contrast to other important gastrointestinal tract pathogens, ADI pathway is far more important than urease for acid resistance and intracellular survival in L. hongkongensis. The gene duplication of the arc gene cassettes could be a result of their functional importance in L. hongkongensis.

Keywords: Laribacter hongkongensis, Acid resistance, Arginine deiminase pathway, Microbe-host interaction
Background

*Laribacter hongkongensis* is a Gram-negative, facultative anaerobic, motile, S-shaped, asaccharolytic, urease-positive bacillus that belongs to the *Neisseriaceae* family of β-proteobacteria [1]. It was first isolated from the blood and thoracic empyema of an alcoholic liver cirrhosis patient in Hong Kong [1]. Recently, it was also recovered from the blood culture of a Korean patient with liver cirrhosis as a result of Wilson’s disease [2]. These cases make chronic liver disease a distinct possible risk factor for invasive *L. hongkongensis* infections, where intestinal mucosal edema and local immunosuppression secondary to portal venous congestion vasculopathy due to liver cirrhosis predisposed the patients to *L. hongkongensis* invasion through the gastrointestinal mucosa. In addition to invasive bactereemic infections, *L. hongkongensis* is also associated with community-acquired gastroenteritis and traveler’s diarrhea [3]. *L. hongkongensis* is likely to be globally distributed, as travel histories from patients suggested its presence in at least four continents: Asia, Europe, Africa and Central America [3-6]. *L. hongkongensis* has been found in up to 60% of the intestines of commonly consumed freshwater fish of the carp family [7,8]. It has also been isolated from drinking water reservoirs and Chinese tiger frogs in Hong Kong and little egrets in Hangzhou [9-11]. Pulsed-field gel electrophoresis and multilocus sequence typing showed that the fish and patient isolates fell into separate clusters, suggesting that some clones could be more virulent or adapted to human [8,12]. These data strongly suggest that this bacterium is a potential diarrheal pathogen that warrants further investigations.

For any gastrointestinal tract pathogen, after transmission through the oral route, the first challenge that the bacterium has to face is the hostile acidic environment of the stomach. When the bacterium invades the intestinal mucosa, it has to survive the attack of submucosal macrophages, which sometimes may be related to its resistance to the acidic environment in endocytic vacuoles. More importantly, for a successful pathogen, the ability of resisting acidic environments is definitely crucial for its survival in different environment and transition from environments to humans. Various gastrointestinal bacteria have developed different mechanisms to overcome this hostile environment and evade host defense. For example, *Helicobacter pylori* and verotoxigenic *Escherichia coli* O157 have developed unique mechanisms to overcome such an acidic environment [13-15]. For *H. pylori*, urease converts urea to carbon dioxide and ammonia and increases the local pH of the bacterium, which is essential for its pathogenesis [16]. During the process of analyzing the *L. hongkongensis* genome, a complete urease cassette, which includes eight open reading frames, encoding three urease structural proteins (UreA, UreB and UreC) and five accessory proteins (UreE, UreF, UreG, UreD and Urel) (Figure 1A), was observed [17]. In addition, two adjacent *arc* gene cassettes, each of them consisting of four genes, *arcA*, *arcB*, *arcC* and *arcD* (Figure 1A), were also found [17]. *arcA*, *arcB* and *arcC* encode the three enzymes, arginine deiminase (ADI), ornithine carbamoyltransferase and carbamate kinase, of the ADI pathway; and *arcD* encodes a membrane bound arginine-ornithine antiporter. These enzymes of the ADI pathway convert L-arginine to carbon dioxide, ATP, and ammonia, which increases the pH of the environment and have been shown to be important for survival of various bacteria in acidic environments [18-21]. We hypothesize that the urease cassette and/or the *arc* gene cassettes are important for *L. hongkongensis* to survive in acidic environments and macrophages. In this study, we tested this hypothesis by systematically knocking out genes in the urease cassette and the two *arc* gene cassettes in *L. hongkongensis* and examining their effects in the survival of the single, double and triple knockout mutants in acidic environment *in vitro*, in macrophages and in a mouse model.

Methods

Ethics statement

The experimental protocols were approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong, in accordance with the Guidelines laid down by the NIH in the USA regarding the care and use of animals for experimental procedures.

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The parental *L. hongkongensis* strain HLHK9, was a clinical isolate from a patient in Hong Kong [3], for which the complete genome has been sequenced [17]. Streptomycin (Sm)-resistant HLHK9 strain was obtained by serial passage of HLHK9 cells on Luria broth (LB) agar with increasing concentrations of Sm, starting at 10 μg/ml, and increased up to 100 μg/ml. Unless stated otherwise, all HLHK9 and its derivative strains used in this study were Sm resistant. HLHK9 and its derivatives were grown in brain heart infusion (BHI) broth or on BHI agar (BHA) plates (BBL, BD) whereas all other *E. coli* strains were grown in LB or on LB agar (LBA) plates (BBL, BD). Media were supplemented with antibiotics (Sigma-Aldrich) when appropriate: ampicillin (Amp) (100 μg/ml), kanamycin (Km) (50 μg/ml), chloramphenicol (Cm) (15 μg/ml), tetracycline (Tet) (12.5 μg/ml) and Sm (100 μg/ml). Growth phase and bacterial cell density were determined by measuring absorbance spectrophotometrically at optical density (OD)600.

Construction of non-polar deletion mutant strains

Primers used for deletion mutagenesis are listed in Table 2. To generate unmarked, non-polar deletion of
ureA, suicide plasmid pDS132 was used for constructing in-frame deletion mutants by homologous recombination [22]. 5′- and 3′-flanking regions of ureA were amplified by PCR from chromosomal DNA of HLHK9, using primers ureA-UF/UR and ureA-DF/DR, respectively, and the individual PCR products were mixed to generate an in-frame deletion pattern of ureA by an overlapping PCR method. The overlapping amplicon containing the in-frame deletion pattern was cloned into pUC19 followed by pDS132, resulting in the final construct of pDS132-ureA, which was electro-transformed into pir-positive E. coli SM10 λ pir [23]. pDS132-ureA was transferred into HLHK9 from transformed SM10 λ pir by bacterial conjugation. Exconjugants having single recombination with suicide vector pDS132 were first selected on BHA with antibiotics (Cm, 15 μg/ml; Sm, 100 μg/ml). After that, the positive enconjugants with single recombination were further cultured and selected.

**Figure 1** Genetic organization of urease gene cassette and the two adjacent arc gene cassettes. A, The open vertical triangles represent the locations of the gene cassettes, and the numbering is according to the sequence of the HLHK9 strain. B, Schematic illustration showing the differences in the sequences of the urease gene cassettes between L. hongkongensis HLHK9 and the naturally urease-negative strain HLHK30. Vertical triangles represent the locations of polymorphic residues, and the numbering is according to the sequence of the HLHK9 strain.

Table 1 Bacterial strains and plasmids used in this study

| Strains or plasmids | Characteristics | Source or reference |
|---------------------|-----------------|--------------------|
| **Strains**         |                 |                    |
| E. coli DH5α        | F, φ80d lacZΔM15, Δ(lacZYA-argF)U169, endA1, recA1, hsdR17(k,R, mK+), deoR, thi-1, supE44, λ, gyrA96(Nal), recA1 | Invitrogen         |
| E. coli SM10(λ pir) | thi thr leu tonA lacI139 relA1 | [23]               |
| L. hongkongensis HLHK1 to HLHK30 | Thirty human strains isolated from patients with community-acquired gastroenteritis in Hong Kong | [3]                |
| HLHK9               | HLHK9 derivative with Sm resistance phenotype, Sm+ | This study         |
| HLHK9ΔureA          | HLHK9 derivative with ureA deletion, Sm+ | This study         |
| HLHK9ΔureC          | HLHK9 derivative with ureC deletion, Sm+ | This study         |
| HLHK9ΔureD          | HLHK9 derivative with ureD deletion, Sm+ | This study         |
| HLHK9ΔureE          | HLHK9 derivative with ureE deletion, Sm+ | This study         |
| HLHK9ΔarcA1         | HLHK9 derivative with arcA1 deletion, Sm+ | This study         |
| HLHK9ΔarcA2         | HLHK9 derivative with arcA2 deletion, Sm+ | This study         |
| HLHK9ΔarcA1/arcA2   | HLHK9 derivative with arcA1 and arcA2 double deletion, Sm+ | This study         |
| HLHK9ΔureA/arcA1/arcA2 | HLHK9 derivative with ureA, arcA1 and arcA2 triple deletion, Sm+ | This study         |
| **Plasmids**        |                 |                    |
| pUC19               | Cloning vector; ori lacZ Amp' | Invitrogen         |
| pCVD442             | Suicide plasmid; R6K ori mob RP4 bia sacB | [25]               |
| pDS132              | Suicide plasmid; R6K ori mob RP4 cat sacB | [22]               |
| pDS132-ureA         | pDS132 carrying 5′- and 3′-flanking regions of ureA for mutagenesis of ureA | This study         |
| Primers for mutagenesis of ureA | Sequence | Restriction site |
|--------------------------------|----------|------------------|
| ureA-UF                        | 5’ GCCTACGAGATTCCTTACGATGGGCTGT | XbaI |
| ureA-UR                        | 5’ GCAGGGAGCGGTGATACACCTCATATTG  |
| ureA-DF                        | 5’ TGGAGGTATCCACCGCCTCCAGCAC     |
| ureA-DR                        | 5’ CCGATGCCTTCTTACGATGGAGCGAGCAG | Sphi |
| Inner-ureA-F                   | 5’ TGCATCTCACCTGCGGTGAG          |
| Inner-ureA-R                   | 5’ TACGGATACGCGGGAATGCA          |

| Primers for mutagenesis of ureC | Sequence | Restriction site |
|--------------------------------|----------|------------------|
| ureC-UF                        | 5’ TCAGAATCCAGGGATCCGGCTCTCCAC  |
| ureC-UR                        | 5’ GTTCCGGGGTACCCAGACGGATCTTG   |
| ureC-DF                        | 5’ TCGCTCGTGGTAAACCCGGGACCTCG   |
| ureC-DR                        | 5’ TGGTGACGGCGGAAGATGTCACCAG    |
| Inner-ureC-F                   | 5’ GTCCGTCCCGAAACCTCAGCGC       |
| Inner-ureC-R                   | 5’ CCTGCGAGGCAAAGGTGATG         |

| Primers for mutagenesis of ureD | Sequence | Restriction site |
|--------------------------------|----------|------------------|
| ureD-UF                        | 5’ GCGAGCTCTCAAAGCCGCACTACGGAAG |
| ureD-UR                        | 5’ GGTACATCGGCTCAACGGCGGATGGC   |
| ureD-DF                        | 5’ CCGTGTGGTACCTGACACCCGGGATGG  |
| ureD-DR                        | 5’ GCGTCGACCCAGTACACCCGACATCATCAG |
| Inner-ureD-F                   | 5’ TACAGCAGGCCTGCTATTG          |
| Inner-ureD-R                   | 5’ GCACGACAGCAGTGCGGAAG         |

| Primers for mutagenesis of ureE | Sequence | Restriction site |
|--------------------------------|----------|------------------|
| ureE-UF                        | 5’ CGGTAGAGGAGCAGCTCTTACGGAAT   |
| ureE-UR                        | 5’ GCATGGTGAACGGCAATCTCCACT     |
| ureE-DF                        | 5’ AGATTGCCCTGCATCCACCTTGCGGAAG |
| ureE-DR                        | 5’ TGCATGACCTCGGCGCAATGTCACCAG |
| Inner-ureE-F                   | 5’ TGCCAGCAGCTACGCGTCAAG        |
| Inner-ureE-R                   | 5’ TGCAGATCTCGGCTTGAGTATG       |

| Primers for mutagenesis of arcA1 | Sequence | Restriction site |
|---------------------------------|----------|------------------|
| LPW14961 (arcA1-UF)            | 5’ CCGCTCGAGTGATGATCCATACGGTGTAACG | Xho1 |
| LPW14962 (arcA1-UR)            | 5’ GTATTGCGGTCTCCTTCAACCGATCAC    |
| LPW14963 (arcA1-DF)            | 5’ GTTGAACAGGACCCTAGTACATCCA     |
| LPW14964 (arcA1-DR)            | 5’ CTAGTCTGATAGCICGCGCCGATCTCTGC |
| LPW16076f (Inner-arcA1-F)      | 5’ ACATGCTAGCCAAAGGTTTG          |
| LPW16077f (Inner-arcA1-R)      | 5’ AAAGCTGCTGCTGCGTACC           |

| Primers for mutagenesis of arcA2 | Sequence | Restriction site |
|---------------------------------|----------|------------------|
| LPW14965 (arcA2-UF)            | 5’ CCGCTCGAGGGATTTATTCCCGGAACAC |
| LPW14966 (arcA2-UR)            | 5’ ACACCCGCGATCGTGCGTCCCTTCTG   |
| LPW14967 (arcA2-DF)            | 5’ CAAGCTAGATCGCGCGGTATG         |
| LPW14968 (arcA2-DR)            | 5’ TGCTCTGATGTAATCGCGGCAAGAAG    |
| LPW16078f (Inner-arcA2-F)      | 5’ ATGCCAAGGGTGCGCGCAAC          |
| LPW16079f (Inner-arcA2-R)      | 5’ AGCGATTCCAGCACCTTC           |
Table 2 Primers used in this study (Continued)

| Primers for real-time qPCR                                                                 |
|------------------------------------------------------------------------------------------|
| LPW21629 (arcA1-F)                                                                         |
| LPW21630 (arcA1-R)                                                                         |
| LPW21631 (arcA2-F)                                                                         |
| LPW21632 (arcA2-R)                                                                         |
| LPW21635 (poxB-F)                                                                          |
| LPW21636 (poxB-R)                                                                          |
| LPW22260 (ureA-F)                                                                           |
| LPW22261 (ureA-R)                                                                           |

Abbreviations: Sm, resistance to streptomycin; Ap, resistance to ampicillin; Cm, resistance to chloramphenicol; Km, resistance to kanamycin; Km, resistance to kanamycin; Tc, resistance to tetracycline; Mu, resistance to mucopeptide; Ery, resistance to erythromycin; Gen, resistance to gentamicin.

Phosphate buffer (containing 10 mM L-arginine) and supernatants were mixed with 0.4 ml of 100 mM potassium by glass beads (Sigma-Aldrich). One milliliter of supernatant was re-suspended in 2 ml extraction solution (2% Triton X-100, 1% SDS, 10 ml overnight culture of test strains were re-suspended in 2 ml urease test broth [26]. The mixtures were incubated at 37°C without shaking. The color change in urease test broth was monitored at 4, 8, 24 and 48 h with the uninoculated reagents were used as positive and blank controls, respectively. The development of an orange color was monitored among the tested strains.

In vitro susceptibility of L. hongkongensis to acid pH
One hundred microliter of overnight cultures of HLHK9 and derivative mutant strains were inoculated into 5 ml of fresh BHI respectively and grown to exponential phase (OD_{600} 0.6 to 0.8), washed with sterile water, and harvested by centrifugation. The pH of the phosphate buffer (PBS, Sigma-Aldrich) was adjusted to 2, 3, 4, 5 and 6 by adding 1 N HCl in the presence or absence of 50 mM urea (for HLHK9, HLHK9 Δ ureA, HLHK9 Δ ureC and HLHK9 Δ ureD, HLHK9 Δ ureE, were grown at 37°C overnight. Bacterial cultures were diluted 1:50 in BHI containing Sm and further cultured at 37°C with shaking, until early-exponential phase (about 0.6 at OD_{600}). One hundred microliter of bacterial cultures was used to inoculate 2 ml urease test broth [26]. The mixtures were incubated at 37°C without shaking. The color change in urease test broth was monitored at 4, 8, 24 and 48 h with the uninoculated urease test as negative control [27].

Qualitative analysis of urease enzyme activity
Thirty human strains, including HLHK9, and mutant strains HLHK9 Δ ureA, HLHK9 Δ ureC, HLHK9 Δ ureD and HLHK9 Δ ureE, were grown at 37°C overnight. Bacterial cultures were diluted 1:50 in BHI containing Sm and further cultured at 37°C with shaking, until early-exponential phase (about 0.6 at OD_{600}). One hundred microliter of bacterial cultures was used to inoculate 2 ml urease test broth [26]. The mixtures were incubated at 37°C without shaking. The color change in urease test broth was monitored at 4, 8, 24 and 48 h with the uninoculated urease test as negative control [27].

Qualitative analysis of ADI activity
A chemical colorimetric method, based on the production of L-citrulline from L-arginine, was used to measure ADI activity of whole-cell lysates of 30 human strains, including HLHK9, and mutant strains HLHK9 Δ arcA1, HLHK9 Δ arcA2 and HLHK9 Δ arcA1/arcA2 [24]. Sucrose-resistant colonies were screened by PCR using primers ureA-UF/DR and Inner-ureA-F/R, which were specific for the deleted sequence. All mutant strains were confirmed by DNA sequencing.

Similarly, non-polar deletion of the ureC, ureD and ureE were constructed respectively as described above (Table 2). Instead of using suicide plasmid pDS132, arcA1, arcA2, arcA1/arcA2 double mutant and ureA/arcA1/arcA2 triple mutant strains were constructed using suicide plasmid pCVD442 [25], and Amp was used as the selection marker.

Specific primers for amplification of arcA1
Specific primers for amplification of arcA2
Specific primers for amplification of arcA2
Restriction sites in the primer sequences are in italic.

Intracellular survival assays in J774 macrophages
J774 macrophages (Sigma-Aldrich) were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) at 37°C in an atmosphere of 5% CO₂. Infection assays were performed as described previously [31,32]. J774 macrophages were seeded to 24-well tissue culture plates at 4 × 10⁵ cells per well and
incubated at 37°C with 5% CO2 for 24 h before infection. Log-phase bacterial cultures (OD600 of 0.6 to 0.7) of the wild type L. hongkongensis HLHK9 and mutants were washed twice with sterile phosphate-buffered saline (PBS) and resuspended in antibiotic-free media. Infection was carried out by inoculating 1 x 10^7 bacterial cells to each well at a multiplicity of infection of about 10:1 and incubated at 37°C for 1 h to allow adhesion and invasion to occur. After that, the culture supernatants were aspirated and the cells were washed three times with sterile PBS. Gentamicin (Sigma-Aldrich) was then added to each well at a concentration of 100 μg/ml and incubated at 37°C for 1 h to kill the extracellular bacteria followed by washing with sterile PBS and replacing the medium with serum-free DMEM containing 25 μg/ml of gentamicin. After 2 and 8 h post-infection, macrophages were lysed with 1% Triton X-100 (Sigma-Aldrich) for CFUs counts. The CFUs recovered from cell lysates after 2 h of phagocytosis were considered as the initial inocula and were used as the baseline values for intracellular survival analysis. CFUs recovered at 8 h were used to calculate the recovery rate of bacterial cells in macrophages. Experiments were repeated in triplicate to calculate the mean of intracellular survival of bacteria.

RNA isolation and real-time quantitative RT-PCR
At 2 h and 8 h post infection, the macrophage monolayers were washed with PBS and lysed with 1% Triton X-100 (Sigma-Aldrich). Total RNA was then extracted respectively using RNase-free Mini kit (Qiagen), followed by treating with RNase-free DNase I (Roche) at 37°C for 20 min. Reverse transcription was performed using the SuperScript III kit (Invitrogen). Real-time RT-PCR assay was performed in ABI7900HT Fast Real Time PCR machine (Applied Biosystems) with FastStart DNA Master SYBR Green 1 Mix reagent kit (Roche), as described by the manufacturer. The sequences of the primers used in the quantitative reverse transcription-PCR (qRT-PCR) were listed in Table 2. The mRNA levels of arcA1 and arcA2 and ureA genes were measured by quantitation of cDNA and the calculated threshold cycle (CT) corresponding to the target gene was calculated as 2^ΔΔCT (Target – CT Reference) and normalized to that of rpoB gene [33].

Survival of L. hongkongensis in mouse model
One hundred microliters of overnight cultures of HLHK9 and mutant strains HLHK9ΔureA, HLHK9ΔarcA1/arcA2 and HLHK9ΔureA/arcA1/arcA2 were inoculated into 5 ml of fresh BHI respectively and grown to exponential phase (OD_600_0.6 to 0.8). The bacteria were harvested by centrifugation at 5,000 g for 15 min and resuspended in PBS to about 10^9 CFUs/ml. Five hundred microliters of bacterial suspension were orally inoculated to groups (n = 5) of 6- to 8-week-old female BALB/c mice which were starved for 6 h previously. Mice were sacrificed 120 min after inoculation and the terminal ileum were removed aseptically and homogenized in 5 ml PBS. Serial dilutions of the homogenates were plated in duplicate on BHA with Sm (100 μg/ml) to determine the number of viable cells [30]. The data were collected from three independent experiments.

PCR amplification and DNA sequencing of arcA1 and arcA2
Extracted DNA from the 30 L. hongkongensis human strains previously isolated from stool specimens of patients with community-acquired gastroenteritis [3], was used as template for amplification of arcA1 and arcA2 genes, using specific primers LPW16076/16077 and LPW16078/16079, respectively. The PCR mixture (25 μl) contained L. hongkongensis DNA, 1× PCR buffer II, 2.0 mM MgCl_2, 200 μM of each dNTPs and 1.0 unit AmpliTaq Gold DNA polymerase (Applied Biosystems). All samples underwent denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min in an automated thermal cycler (Applied Biosystems). Five microliters of each amplified product was electrophoresed in 2% (wt/vol) agarose gel and Tris-borate-EDTA buffer, with molecular size marker (GeneRuler 50-bp DNA ladder; Fermentas) in parallel, at 100 volts for 1 h. Five PCR products were randomly selected, gel-purified and sequenced with an ABI Prism 3700 DNA Analyzer (Applied Biosystems), using the PCR primers.

Statistical analysis
Statistical analyses were performed using Prism 5.01 (GraphPad). CFU counts were logarithmically transformed prior to analysis. Unless stated otherwise, data generated were expressed as mean +/- standard error of the mean (SEM). Statistically significance was calculated using the unpaired student's t-test. p < 0.05 was considered statistically significant (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Results
Examination of L. hongkongensis strains for urease activity
With the exception of native urease-negative L. hongkongensis HLHK30, the urease test broth incubated with all human strains, including HLHK9, began to turn pink after 4 h (Figure 2A), and the color became more intense after 24 h of incubation. Similar to the natural urease-negative strain HLHK30, mutant strains HLHK9ΔureA, HLHK9ΔureC, HLHK9ΔureD and HLHK9ΔureE elicited no color change after prolonged incubation (Figure 2A). These results indicated that these four urease genes were all essential for the urease enzyme activity.
Examination of *L. hongkongensis* strains for ADI activity

In the qualitative assay, similar to the positive control (citrulline standard), cellular extracts prepared from all 30 human strains, including wild type *L. hongkongensis* HLHK9, also generated an orange color, confirming that citrulline was being produced (Figure 2B). Cell extracts from both single knockout mutant strains, HLHK9ΔarcA1 and HLHK9ΔarcA2, also yielded an orange color, whereas deletion of both arcA1 and arcA2 abolished the ADI activity (Figure 2B). These results showed that both the arcA1 and arcA2 genes encode functional ADI enzymes, which could complement the functions of each other.

In vitro susceptibility of urease-negative mutants to acid

To study the role of the two arc loci of *L. hongkongensis* under acidic conditions, wild type *L. hongkongensis* HLHK9, HLHK9ΔarcA1, HLHK9ΔarcA2, HLHK9ΔarcA1/arcA2 were exposed to different acidic pHs (pH 2 to 6) in the presence and absence of 50 mM of L-arginine, respectively. In the absence of L-arginine, survival of the three mutants were similar to that of HLHK9 at ≥pH 4, and they became susceptible at ≤pH 3 (data not shown). In the presence of L-arginine, the survival of the three mutants were similar to that of HLHK9 at ≥pH 4, and they became susceptible at ≤pH 3 (data not shown). In the presence of L-arginine, wild type *L. hongkongensis* HLHK9, HLHK9ΔarcA1 and HLHK9ΔarcA2 survived well under all tested pHs, suggesting that the two copies of the arcA gene performed complementary functions in *L. hongkongensis* (Figure 3B). On the other hand, the survival of HLHK9ΔarcA1/arcA2 decreased about 2-log at pH 4 (p < 0.05) and it was barely recovered at pH 2 and 3 (p < 0.01) (Figure 3B). This indicated that the ADI pathway played a crucial role in the survival of *L. hongkongensis* under acidic conditions.

In vitro susceptibility of urease- and ADI-negative triple knockout mutant to acid

Given the above results that both the urease and ADI pathway contribute towards the overall acid tolerance of *L. hongkongensis*, we constructed a triple knockout mutant strain HLHK9ΔureA/arcA1/arcA2 and compared its survival abilities with HLHK9, HLHK9ΔureA and HLHK9ΔarcA1/arcA2 under different acidic conditions in the presence of 50 mM each of L-arginine and urea.
The parental and mutant strains displayed similar susceptibilities at pH 5 (Figure 3C). At pH 4, the survival count of HLHK9ΔureA was similar to that of HLHK9 but there was about 2-log reduction in that of HLHK9ΔarcA1/arcA2 (p < 0.01) and HLHK9ΔureA/arcA1/arcA2 (p < 0.001) (Figure 3C), and the reduction trend became more pronounced after 3 and 5 h incubation (Figure 3D). At pH 2 and 3, the survival counts of HLHK9ΔureA started to decrease (p < 0.05), whereas there were dramatic decreases in the survival counts of HLHK9ΔarcA1/arcA2 (p < 0.001) and triple knockout mutant HLHK9ΔureA/arcA1/arcA2 strains, which were almost completely killed (p < 0.001) (Figure 3C). These showed that the ADI pathway of L. hongkongensis played a more important role than the urease in resisting acidic environments.

**Intracellular survival in J774 macrophages and mRNA expression level analyses**

Survival of wild type L. hongkongensis HLHK9, HLHK9ΔureA, HLHK9ΔarcA1/arcA2 and HLHK9ΔureA/arcA1/arcA2 in J774 macrophages were shown in Figure 4A. Survival of HLHK9ΔureA/arcA1/arcA2 and HLHK9ΔarcA1/arcA2 in macrophages were markedly decreased (p < 0.001 and p < 0.01 respectively) but that of HLHK9ΔureA was slightly decreased (p < 0.05), compared to wild type L. hongkongensis HLHK9. The decrease of survival was more prominent in HLHK9ΔureA/arcA1/arcA2, compared to HLHK9ΔarcA1/arcA2 (p < 0.05) and HLHK9ΔureA (p < 0.01); and in HLHK9ΔarcA1/arcA2, compared to HLHK9ΔureA (p < 0.05). Given the above results, we further investigated the expression level of ADI genes (arcA1 and arcA2) and ureA gene of wild type L. hongkongensis HLHK9 survived in macrophages using real-time quantitative RT-PCR assay. At 8 h post infection, the mRNA levels of arcA1, arcA2 and ureA genes were markedly increased compared to those at 2 h post infection (p < 0.05, p < 0.01 and p < 0.05 respectively) (Figure 4B).

**Survival of L. hongkongensis strains in BALB/c mice**

To further investigate the role of urease and ADI pathway in acid tolerance of L. hongkongensis, we compared the survival ability of HLHK9, mutant strains HLHK9ΔureA, HLHK9ΔarcA1/arcA2 and HLHK9ΔureA/arcA1/arcA2.
after transit through the stomach of mice. Using this mouse model, HLHK9ΔureA exhibited similar survival abilities as HLHK9 (Figure 5). In contrast, the viable counts of HLHK9ΔarcA1/arcA2 and HLHK9ΔureA/arcA1/arcA2 were reduced by 1.2-log and 1.3-log respectively, compared to that of HLHK9 (p < 0.01) (Figure 5). This also indicated that the ADI pathway played a more significant role than urease in the survival of L. hongkongensis under the acidic conditions encountered during passage through the mouse gastric environment.

**PCR amplification and DNA sequencing of arcA1 and arcA2**

A specific 739-bp fragment of arcA1 and a specific 712-bp fragment of arcA2 of L. hongkongensis were amplified from the DNA extracts of all 30 human strains, indicating that both arcA1 and arcA2 were present in all 30 human strains. DNA sequencing of the PCR products from five randomly selected L. hongkongensis strains confirmed that the amplified products were arcA1 and arcA2 respectively. Sequence analyses showed that there were 1 to 5 nucleotide differences and one amino acid difference between the 739-bp fragments and the deduced amino acid sequences of the arcA1 genes from these five selected strains and the corresponding region of HLHK9. Similarly, there were 1 to 4 nucleotide differences but no amino acid difference between the 712-bp fragments of the arcA2 genes from these five strains and the corresponding region of HLHK9. Sequence analysis also revealed that most of the conserved residues were present in the partial fragments of arcA1 and arcA2, compared to ADI sequences of other bacteria.
Discussion
We showed that the arc gene cassettes are more important than the urease gene cassette for acid resistance and survival in macrophages in *L. hongkongensis*. Although both urease and arc gene cassettes have previously been reported to play roles in acid resistance in bacteria, urease function appears to be more important in gastrointestinal tract bacteria such as *H. pylori*, *Yersinia enterocolitica* and *Klebsiella pneumoniae* [16,30,34]. In fact, the mechanisms of acid resistance are similar in both reactions, which result in production of ammonia, thereby increasing the pH of the immediate environment of the bacterium. As for survival in macrophages, ADI pathway has been shown to contribute to survival in macrophages in *Salmonella Typhimurium* [32], but not in *Listeria monocytogenes* [29]; and urease has been shown to contribute to survival in macrophages in *H. pylori* [35], but not in *Brucella suis* and *Brucella abortus* [30,36]. To the best of our knowledge, the present study is the first to compare the relative importance of these two acid resistance and intracellular survival mechanisms using *in vitro* and *in vivo* models, although these two gene cassettes are present in many gastrointestinal tract bacteria, such as *Y. enterocolitica* and *Enterobacter cloacae*. By constructing a series of urease knockout mutants, we found that both structural and accessory genes in the urease gene cassette are crucial for the urease activity; which is in line with previous studies performed in other bacterial species [15,30,37]. Contrary to our initial hypothesis, we observed only a small reduction in survival abilities of the urease knockout mutants in acidic media (pH 2 and 3) and macrophages as well as during gastric passage in the mouse model. This is consistent with our previous recovery of a strain of urease-negative *L. hongkongensis* (HLHK30) from an 84-year old male with gastroenteritis. Sequencing of the urease cassette of HLHK30 showed that all eight of the component genes were present with no deletions or frame shift mutations; although there were a number of polymorphic sites that resulted in amino acid changes compared to gene homologues present in HLHK9 (Figure 1B). On the other hand, the ADI-deficient mutant HLHK9ΔarcA1/arcA2 showed marked reduction in survival abilities in acidic media and macrophages as well as in the mouse model, indicating that arc gene cassettes play a more important role than urease gene cassettes for acid resistance in *L. hongkongensis*. In fact, the survival abilities of the triple knockout mutant strain HLHK9ΔureA1/arcA1/arcA2 were only marginally lower than those of the ADI-deficient double mutant strain HLHK9ΔarcA1/arcA2 in acidic media and macrophages, and both mutant strains had equivalent survival abilities in the mouse model, which further supports the conclusion that ADI play a more important role.

The gene duplication of the arc gene cassettes could be a result of their functional importance in *L. hongkongensis*. One of the important mechanisms of virulence evolution in bacteria and fungi is gene duplication [38-40]. *L. hongkongensis* is the only bacterium known to possess two adjacent arc gene cassettes. The *L. hongkongensis* mutant strain containing deletions of the arcA genes in both arc cassettes exhibited a marked reduction in survival abilities compared to the mutant strains containing single deletion of either one of the two arcA genes, indicating that both arc gene cassettes are functional and contribute to acid resistance. Phylogenetic analysis showed that the two copies of arc in *L. hongkongensis* are clustered in all the four trees constructed using arcA, arcB arcC and arcD [41]. This strongly suggests that the two arc gene cassettes result from a gene cassette duplication event. Interestingly, in our previous study on differential gene expression in *L. hongkongensis* at different temperatures, it was observed that the two copies of argB, encoding two isoenzymes of N-acetyl-L-glutamate kinase from the arginine biosynthesis pathway, which have distinct biochemical properties, are also clustered phylogenetically [17]. This indicates that these two copies of argB probably also arose as a result of gene duplication. Subsequent evolution enabled the two copies of argB to adapt to different temperatures and habitats. These coincidental findings of gene duplication in two different pathways of arginine metabolism, enabling the bacterium to better adapt to different environmental conditions, argB for temperature adaptation and arc gene cassette for acid resistance, is intriguing.

The present study further strengthened the feasibility of using a conjugation mediated gene deletion system based on a suicide vector in the *Neisseriaceae* family of β-proteobacteria, a strategy which has been widely used in γ-proteobacteria, such as *E. coli*, *Salmonella Typhimurium* and *Vibrio cholera* [22,42,43]. In our previous studies on plasmid transformation and gene expression system in *L. hongkongensis*, we observed that plasmids commonly used for expression systems in *E. coli* did not replicate in *L. hongkongensis* [44]. Therefore, an *E. coli*- *L. hongkongensis* shuttle vector, based on a *L. hongkongensis* plasmid backbone and origin of replication, was constructed [44]. In our subsequent gene deletion experiments in *L. hongkongensis*, we used a pBK-CMV plasmid that harbored 1000 bp of genomic upstream and downstream of the target gene, but lacked the target gene, which was transformed into *L. hongkongensis*. This gene deletion system was successfully used to delete several *L. hongkongensis* genes, such as the flagG flagellar gene. However attempts to delete the ureA, ureB, ureC and ureI genes were all unsuccessful (unpublished data). Therefore, the present gene deletion system, which was first used in *E. coli* [42], and also recently used in
Chromobacterium violaceum, another pathogenic bacterium of the Neisseriaceae family [45], was used for knocking-out genes from the urease and arc gene cassettes. Further experiments will elucidate whether this gene deletion system is also useful for knocking out genes in other important bacteria of the Neisseriaceae family, such as the Neisseria gonorrhoeae and Neisseria meningitidis.

Conclusions

ADI pathway is far more important than urease for acid resistance and intracellular survival in L. hongkongensis. The gene duplication of the arc gene cassettes could be a result of their functional importance in L. hongkongensis.

Abbreviations

Bp: Base pairs; DNA: Deoxyribonucleic acid; µg: Microgram; ml: Milliliter; µl: Microliter; M: Molar; min: Minutes; PCR: Polymerase chain reaction; h: Hour; DMEM: Dulbecco's modified eagle medium; EDTA: Ethylenediaminetetraacetic acid; SDS: Sodium dodecyl sulfate; NaCl: Sodium chloride.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

PCYW and JLLT conceived the study. PCYW, JLLT and LX performed the experiments. LX, JLLT and PCYW analyzed the data; RMW, BK and SKPL corrected the manuscript; all authors read and approved the final manuscript.

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