Putative Riemerella anatipestifer Outer Membrane Protein H Affects Virulence

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Riemerella anatipestifer causes serious contagious disease in ducks, geese, and other fowl. However, as a harmful pathogen causing significant economic losses in the poultry industry, R. anatipestifer is still poorly understood for its pathogenesis mechanisms. In a previous study, we developed an indirect ELISA method for detecting R. anatipestifer infection using B739_0832 protein, a putative outer membrane protein H (OmpH) that is conserved among different serotypes of R. anatipestifer. Although OmpH in some pathogenic bacteria, such as Pasteurella, has been reported as a virulence factor, it is still not clear whether B739_0832 protein contributes to the virulence of R. anatipestifer.

In this study, we confirmed that B739_0832 protein in R. anatipestifer localizes to the outer membrane. We constructed a B739_0832 deletion mutant strain (ΔB739_0832) and assayed various effects from the deletion of B739_0832. ΔB739_0832 strain had a similar growth rate to wild-type R. anatipestifer CH-1. However, the survival rate of ducklings in 10 days after infection from ΔB739_0832 strain was 50%, whereas no ducklings survived from wild-type R. anatipestifer infection. Furthermore, the median lethal dose (LD50) of the ΔB739_0832 strain was approximately 150 times higher than that of the wild-type strain. Pathology examinations on infected ducklings found that, at 36 h after infection, bacterial loads in blood, liver, and brain tissues from ΔB739_0832-infected ducklings were considerably lower than those from wild-type infected ducklings. These results demonstrate that the B739_0832 protein contributes to the virulence of R. anatipestifer CH-1.

Keywords: outer membrane protein, virulence factor, OmpH, B739_0832, Riemerella anatipestifer

INTRODUCTION

Riemerella anatipestifer is a Gram-negative, rod-shaped bacterium in the Flavobacteriaceae family, Riemerella genus (Segers et al., 1993). Riemerella anatipestifer is one of the most serious bacterial threats harming mostly the duck industry, but R. anatipestifer infection has also been reported in other waterfowl worldwide, causing heavy economic losses (Wang et al., 2010; Hu et al., 2012).
**Materials and Methods**

**Bacterial Strains, Plasmids, and Growth Conditions**

Bacterial strains, plasmids, and primers used in this study are listed in Tables 1, 2, respectively. *Riemerella anatipestifer* CH-1 (RA CH-1) strain was used as the wild-type strain, and all other strains used were derived from RA CH-1. *R. anatipestifer* was grown in Tryptic Soy Broth (TSB) or Giotalli-Cantoni Broth (GCB) at 37°C with shaking (Liu et al., 2017). GCB agar plates were prepared by supplementing GCB with 1.5% agar. Alternatively, *R. anatipestifer* was also grown on Luria-Bertani (LB) agar plate supplemented with 5% sheep blood. When necessary, appropriate concentrations of antibiotics were added to the media: ampicillin (Amp, Sigma-Aldrich, 100 µg/ml), chloramphenicol (Cm, Sigma-Aldrich, 30 µg/ml), kanamycin (Kan, Sigma-Aldrich, 50 µg/ml), and cefoxitin (Cfx, Sigma-Aldrich, 1 µg/ml).

**Construction of Clean B739_0832 Deletion Mutant Strain (ΔB739_0832)**

A ΔB739_0832 mutant strain was constructed by using the natural transformation method as previously described (Liu et al., 2017). Briefly, about 700-bp fragments upstream and downstream of B739_0832 gene were amplified using primer pairs ΔB739_0832 up-arm P1 and P2, ΔB739_0832 down-arm P1 and P2, respectively (Table 2). The amplified upstream and downstream fragments were connected to a pLMF02:sacB plasmid using ligation (Tian et al., 2020). The recombined plasmid was further processed with restriction enzymes Nhel and PstI to extract a DNA fragment that has a Cfx-sacB cassette.

**TABLE 1 | Strains and plasmids used in this study.**

| Strains | Genotype or description | Source or references |
|---------|-------------------------|---------------------|
| R. anatipestifer CH-1 | Kan<sup>R</sup> | Laboratory collection |
| ΔB739_0832 | R. anatipestifer CH-1 ΔB739_0832 | This study |
| CΔB739_0832 | R. anatipestifer CH-1 ΔB739_0832 carrying pLMF02:B739_0832 plasmid | This study |

| Plasmids | Genotype or description | Source or references |
|----------|-------------------------|---------------------|
| pLMF02 | Derivative of pPM5, Amp<sup>R</sup>, Cfx<sup>R</sup> | Liu et al., 2017 |
| pLMF02:sacB | Derivative of pLM02, Amp<sup>R</sup>, Km<sup>R</sup>, Cfx<sup>R</sup> | Tian et al., 2020 |
| pLMF02:B739_0832 | pLMF02 carrying B739_0832 from R. anatipestifer CH-1, Cfx<sup>R</sup> | This study |

Amp<sup>R</sup>, ampicillin resistance; Kan<sup>R</sup>, kanamycin resistance; Cfx<sup>R</sup>, cefoxitin resistance.
cassette in the center flanked by the amplified upstream and downstream fragments. The DNA fragment was purified using a Universal DNA Purification kit (TIANGEN™, Beijing, China) and served as donor DNA. Wild-type \textit{R. anatipestifer} CH-1 was transformed with the purified DNA fragment, and cefoxitin-resistant, sucrose-sensitive recombinants were scored. Another DNA fragment fusing the upstream and downstream fragments were produced using the overlap PCR method. The scored cefoxitin-resistant, sucrose-sensitive recombinant strain was further transformed with the upstream–downstream overlap DNA fragment; the \(\Delta_B739\_0832\) mutant strain was scored as cefoxitin-sensitive and sucrose-resistant recombinants. The \(\Delta_B739\_0832\) mutant strain was confirmed by PCR amplification and sequencing (Supplementary Figure 1).

A complementary strain (C\(\Delta_B739\_0832\)) was prepared by transforming the \(\Delta_B739\_0832\) mutant strain with a \(\Delta_B739\_0832\) expressing plasmid (pLMF02: \(\Delta_B739\_0832\)). Briefly, \(B739\_0832\) gene was cloned into pLMF02—a pPM5 derivative—plasmid (Tian et al., 2020). The \(\Delta_B739\_0832\) mutant strain was then transformed with the pLMF02: \(\Delta_B739\_0832\) plasmid using the natural transformation method as described in Liu et al. (2017) and selected for cefoxitin resistance colonies. Expression of \(B739\_0832\) in the complemented strain was confirmed by Western blot.

### Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was used to measure transcription expression levels of \(B739\_0832\) and flanking genes (Liu et al., 2016). Total RNA of the wild-type strain and the \(\Delta_B739\_0832\) mutant strain was extracted from cell cultures at OD\(_{600}\) = 1.0 using RNeasy pure Cell/Bacteria kits (TIANGEN™, Beijing, China). To eliminate DNA contamination, all extracted total RNA samples were treated with RNase-free DNase I (40 U/mg RNA, Takara, China) and purified using RNeasy Mini Kits (Qiagen, Germany). HiScript reverse transcriptase (Vazyme, China) was used to generate cDNA in accordance with manufacturer’s instructions. qRT-PCR was performed using SYBR Green Master Mix (Bio-Rad, United States) and primers listed in Table 2. The expression level of 16S rRNA was used as an internal control. Measurements were performed with three separate cell samples for each gene and were replicated in triplicate. Data were analyzed with a normalized gene expression method \(\left(2^{-\Delta\Delta C_t}\right)\) as previously described (Pfaffl, 2001).

### Growth Rate Determination

Bacterial growth rates were determined as previously described (Luo et al., 2015). Briefly, each strain was activated on an LB plate supplemented with 5% sheep blood overnight at 37°C. A single colony from each strain was inoculated into 5 ml of TSB and cultured at 37°C with agitation for 10 h. Subsequently, each culture was adjusted to an OD\(_{600}\) of 0.05 in 20 ml of fresh TSB and grown at 37°C with shaking at 180 rpm. OD\(_{600}\) for each culture was determined at every 1 h for 18 h.

### Total Membrane Extraction, Separation of Inner Membrane and Outer Membrane, and Western Blot

\textit{Riemerella anatipestifer} total membrane, inner membrane, and outer membrane were extracted and separated based on methods previously described by Hu D. et al. (2019), Thein et al. (2010), and Osborn and Munson (1974). Cells were grown in 1 L TSB to OD\(_{600}\) ≈ 3 at 37°C. Chloramphenicol was then added to the culture at 1 mg/ml final concentration to stop protein synthesis and cells with chloramphenicol were agitated for another hour to ensure that all localization processes are completed. Cells were harvested and resuspended in 10 ml of 0.2 M Tris–HCl, pH 8.0, 1 M sucrose, and 1 mM EDTA, and lysozyme was added to a final concentration of 1 mg/ml. The cells were incubated on ice for 10 min. Spheroplast was prepared by slowly mixing 40 ml ice-cold H\(_2\)O into the cell suspension. The cells were collected by centrifugation at 200,000 \(\times\) g for 45 min at 4°C. The cell pellet was resuspended in 10 ml of ice-cold 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.2 mM DTT, and 1 mg/ml

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**TABLE 2** | Primers used in this study.

| Primers          | Sequence (5′ → 3′)                        | Organism or references               |
|------------------|------------------------------------------|-------------------------------------|
| 16S rRNA P1      | CGAAAAGTGAATAAGTTGAACCACT                 | Zhang et al., 2017                  |
| 16S rRNA P2      | GCGAGCACCCTGAAAATGTGAC                  | This study                          |
| B739_0831 P1     | CTCAATACAAAGGCAAGAA                     |                                     |
| B739_0831 P2     | TTCCCTCTGTCTTAGTGGCT                    |                                     |
| B739_0833 P1     | GCGACCATAAGGACATG                      |                                     |
| B739_0833 P2     | GGAATACGCTATGTTACCTCTGAC                |                                     |
| \(\Delta_B739\_0832\) uparm P1 | CTAACCTAGCCGACTTGCTAGCGGATTG          |                                     |
| \(\Delta_B739\_0832\) uparm P2 | GGGGCTACAATATGAATATAAGTTGTTATTTTAGG     |                                     |
| \(\Delta_B739\_0832\) downarm P1 | ACGCGCTGACTTAGTGAAAATCTATAGAACGCCAC    |                                     |
| \(\Delta_B739\_0832\) downarm P2 | AACTGCAAGATACCTAATTGTGGCTG           |                                     |
| \(\Delta_B739\_0832\) overlap uparm P1 | CTTTGCTATTGGATTATGAAACTATAG           |                                     |
| \(\Delta_B739\_0832\) overlap uparm P2 | GCTTTATAGATATTTTTCACTTTAATAAATGAATTTTATAG |                                     |
| \(\Delta_B739\_0832\) overlap downarm P1 | TAAAAATAACTTTATTTATTTTAAGTTGAAATATCTATAAAGGCCAC |                                     |
| \(\Delta_B739\_0832\) overlap downarm P2 | TTAACCTAATTGTCCCTGAC                   |                                     |
DNase. The cells were lysed by passing through a French Press twice at 10^8 Pa. The sample after French Press was centrifuged at ~3,000 × g for 15 min to remove cell debris. The supernatant was ultracentrifuged at 120,000 × g for 2 h at 4°C to collect total membrane. The total membrane pellet was resuspended in 1 ml of ice-cold 10 mM Tris–HCl, pH 7.5, 15% sucrose (w/v), 5 mM EDTA, and 0.2 mM DTT. Inner membrane and outer membrane were separated on a sucrose step gradient (1 ml of 55% sucrose and 2.25 ml each of 50, 45, 35, and 30% sucrose). The total membrane suspension was placed on top of the sucrose gradient and centrifuged at 250,000 × g for 12 h at 4°C. The outer membrane (lower band, higher density) and inner membrane (upper band, lower density) were extracted by syringes. Each sample was washed three times with 1 ml of Tris buffer (10 mM Tris–HCl, pH 7.5, and 1 mM EDTA) and centrifuged at 100,000 × g for 20 min to collect the membrane samples. The samples were resuspended in SDS-PAGE sample loading buffer for analysis. Whole cell samples were prepared by collecting 2 ml of cells and resuspended in 1 ml of SDS-PAGE sample loading buffer.

His-tagged B739_0832 expression plasmid [pET32a(+)-ompH] was obtained in our previous study (Gao et al., 2016). His-tagged B739_0832 protein was purified and used to generate rabbit polyclonal antibody, as described before (Zhang et al., 2017). Whole cell, total membrane, inner membrane, and outer membrane of R. anatipestifer samples were analyzed using Western blot assay; OmpA was used as an outer membrane protein reference, TonB was used as an inner membrane protein reference, and RecA was used as a cytoplasmic protein reference (Thein et al., 2010; Liao et al., 2015; Xu et al., 2020).

Assessment of LD_{50}

Groups of 3-day-old Cherry Valley ducklings were used to assess LD_{50} of ΔB739_0832 and wild-type strains. Ducklings were divided into 16 groups (10 ducklings per group, 160 total): 5 groups were challenged with wild-type R. anatipestifer CH-1, 5 groups with ΔB739_0832, 5 groups with CΔB739_0832, and 1 group with saline control. The wild-type group was intramuscularly injected at a dose of 10^6 to 10^{10} CFU; the ΔB739_0832 group was injected at a dose of 10^7 to 10^{11} CFU, the CΔB739_0832 group was injected at a dose of 10^6 to 10^{10} CFU, and the control group was injected with 1 ml of sterile phosphate-buffered saline (PBS). LD_{50} values were calculated using SPSS 23.0 (Arambašić and Randhawa, 2014).

Determination of Bacterial Load in Infected Duck Tissues

Three groups of 3-day-old Cherry Valley ducklings (15 ducklings per group) were intramuscularly injected with wild-type, ΔB739_0832, or CΔB739_0832 at a dose of 10^9 CFU, respectively. After challenge, blood, liver, heart, brain, and spleen tissues samples were collected at 6, 12, 24, 36, 48, and 72 h. Three ducklings were randomly selected for sacrifice at each time point. The organ samples were weighed and transferred into tubes each containing 3 ml of sterilized PBS. After homogenization, the tubes were centrifuged for 5 min at 2,000 × g to remove cell debris, the supernatant of each tube was serially diluted with PBS, and 50 μl of each serial dilution was plated on a TSA plate. TSA plates were incubated at 37°C overnight for bacterial count.

Assessment of Duck Survival Rate

Forty 3-day-old Cherry Valley ducklings were randomly divided into four groups (10 ducks per group). One group was challenged with a dose of 10^{10} CFU wild type, one with ΔB739_0832, one with CΔB739_0832, and the fourth group as a control was intramuscularly injected with equal volume (1 ml) PBS. The ducklings were observed for 10 consecutive days after the challenge. All ducklings were indoor and had access to plenty of food and water. Survival rates were calculated each day as the proportion of living ducklings accounted for the initial duckling counts.

Bacterial Adhesion Assay

Bacterial adhesion assay was performed with duck embryo fibroblast (DEF) cells as previously described (Hu et al., 2011). Briefly, each well in a 24-well tissue culture plate was seeded with 1 ml of 2 × 10^5 cells/ml DEF cells in Dulbecco’s Modified Eagle Medium (DMEM; Biowest, France) and incubated at 37°C with 5% CO_2 for 18 h. After confirming that there was at least 95% confluence and has no contamination, each well was infected with 10^7 CFU R. anatipestifer (multiplicity of infection MOI = 50:1). The plates were then incubated at 37°C with 5% CO_2 for another 1.5 h. After incubation, the wells were washed three times with PBS to remove non-adherent bacteria and then incubated at 37°C with 5% CO_2 for 10 min in the presence of 0.25% trypsin (100 μl/well) to release the DEF cells from the wells. Serial 10-fold dilutions were prepared from the cell suspensions and 50 μl of each dilution was plated onto TSA plates to determine adhered bacteria counts. Each assay was performed in triplicate and replicated three times.

Bacterial Invasion Assay

Bacterial invasion assay was also performed with DEF cells as previously described (Hu et al., 2011). DEF cells were grown in 24-well tissue culture plates and then infected with R. anatipestifer the same way as the adhesion assay described above. For the invasion assay, after the infection incubation, 100 μg/ml gentamicin was added to each well and the plate was incubated for an additional 1 h at 37°C to kill all extracellular bacteria. After the extra incubation, the wells were washed three times with PBS and treated with 100 μl of 1% Triton X-100 to lyse the DEF cells. Lysed cells were homogenized. Serial 10-fold dilutions were prepared from the cell lysate and 50 μl of each dilution was plated onto TSA plates to determine invasive bacteria counts. Each assay was performed in triplicate and replicated three times.

Statistical Analysis and Ethics Statement

Statistical analysis was performed with GraphPad Prism 7.0 for Windows (GraphPad Software Inc., San Diego, CA, United States) (Hu Y. C. et al., 2019). Significance of difference between two data sets was evaluated using Student’s t-test, and a value of p < 0.05 was considered significant (Mishra et al., 2019).
Three-day-old Cherry Valley ducklings were procured from Sichuan Agricultural University duck farm and kept under appropriate conditions with a 12-h light/dark cycle and free access to food and water during this study. All ducks were handled in strict adherence to the recommendations of the local animal welfare bodies and Sichuan Agricultural University (No. XF2014-18). The animal-use procedures were approved by the Animal Ethics Committee of Sichuan Agricultural University (Approval No. 2016-015).

RESULTS

Bioinformatics Analysis of B739_0832 Locus in RA CH-1 Strain

In the National Center for Biotechnology Information (NCBI) database, the B739_0832 locus in RA CH-1 has been identified as a 501-base-pair ORF, which encodes a 166-amino acid protein, with a molecular mass of about 18 kDa. The B739_0832 protein has been annotated as an OmpH family outer membrane protein. We analyzed the amino acid sequences of B739_0832 proteins from all sequenced R. anatipestifer strains using the protein–protein Basic Local Alignment Search Tool (BLASTP). The sequence alignment results from BLASTP indicated over 95% identity among different R. anatipestifer strains.

OmpH (also known as Skp) proteins in some Gram-negative bacteria have been shown to be either outer membrane protein or chaperone proteins for outer membrane proteins. We compared the amino acid sequence of B739_0832 protein from R. anatipestifer strains with the OmpH (Skp) sequences from E. coli, Salmonella, and Pasteurella using Clustal Omega, a multiple sequence alignment tool from EMBL-EBI (Madeira et al., 2019; Supplementary Figure 2). The amino acid sequence alignment results indicated that the B739_0832 protein is closer in evolution to OmpH from Pasteurella than those from E. coli or Salmonella. We also analyzed the hydrophobicity properties of the B739_0832 protein using ExPASY software from SIB Swiss Institute of Bioinformatics (results not shown). The results indicated that, of the 166 residues in B739_0832 protein, 63 of them are hydrophobic, and both carboxyl and amino ends of the protein show more hydrophobicity than the middle portion. The results are consistent with outer membrane protein propensities.

Construction and Characterization of ΔB739_0832 Strain and Complemented Strain CΔB739_0832

To elucidate functions of B739_0832 in RA CH-1, we constructed a B739_0832 clean deletion strain (ΔB739_0832) and a complemented strain (CΔB739_0832) using the natural transformation method as described in “Materials and Methods.” Deletion of the B739_0832 gene was confirmed by PCR amplification using primers flanking the locus. PCR amplification of the 16S rRNA gene was performed at the same time as a positive control. The confirmed B739_0832 deletion mutant strain was named as ΔB739_0832. The B739_0832 gene from the RA CH-1 wild-type strain was amplified and cloned into a pLM02 vector plasmid to generate a recombination plasmid pLMF02:B739_0832. This recombination plasmid was introduced into the ΔB739_0832 strain by conjugation to yield the complemented strain CΔB739_0832.

We compared the growth rates of wild-type RA CH-1, ΔB739_0832, and CΔB739_0832 strains. The growth rate of the mutant strain ΔB739_0832 showed no significant difference from that of the wild-type strain and the complemented strain (Figure 1A). We further tested transcription levels of genes upstream (B739_0831) and downstream (B739_0833) of the B739_0832 locus. As shown in Figure 1B, the transcription levels of upstream and downstream genes were not affected by the deletion of B739_0832, which indicates that deletion of B739_0832 did not cause polar effect.

ΔB739_0832-Infected Ducklings Have Increased Survival Rate Than Those Infected by Wild Type

To determine the impact of B739_0832 deletion on RA CH-1 virulence, we measured the mortality rates in ducklings caused by RA CH-1, ΔB739_0832, and CΔB739_0832 strains. Groups of 3-day-old Cherry Valley ducklings were infected by one of these three strains at a dose of 10^{10} CFU and were observed for 10 days. At day 7 after infection, ducklings infected by ΔB739_0832 had about 70% survival rate, whereas ducklings infected by wild-type RA CH-1 or complemented strain CΔB739_0832 only had about 10% survival rate. After 10 days, no ducklings survived from infection by RA CH-1, and only 10% survived infection from CΔB739_0832, whereas 50% ducklings survived from infection by ΔB739_0832. The wild-type and complementary groups had similar patterns; about half of the ducklings in these two groups did not survive for more than 5 days. However, survival rates in the ΔB739_0832 group were significantly different from those in the wild-type and complementary groups (Figure 3).
To further quantify the impact of \( B739\_0832 \) deletion on RA CH-1 virulence, we measured half lethal dose (LD\(_{50}\)) of these three strains. The LD\(_{50}\) of RA CH-1 was \( 3.98 \times 10^8 \), which was about 150 times lower than that of the \( \Delta B739\_0832 \) strain \( (6.09 \times 10^{10}) \). The LD\(_{50}\) of complemented strain \( C\Delta B739\_0832 \) was \( 7.76 \times 10^8 \), which was similar to wild type.

**Deletion of \( B739\_0832 \) Gene Decreased *Riemerella anatipestifer* Adhesion and Invasion in Duck Embryo Fibroblast Cells**

To assess whether deletion of \( B739\_0832 \) gene affected adherence and invasion activities of *R. anatipestifer*, the activities of wild-type, \( \Delta B739\_0832 \), and \( C\Delta B739\_0832 \) strains were measured using DEF cells. DEF cells were infected at MOI of 50:1; the \( \Delta B739\_0832 \) strain had \( 8.77 \pm 1.17 \times 10^3 \) CFU/well adhesion activity, which was approximately threefold lower than that of wild type \( (3.44 \pm 0.16 \times 10^4 \text{ CFU/well}; \text{Figure 4A}) \). Bacterial

**FIGURE 1** | Characterization of mutant strain \( \Delta B739\_0832 \) and complemented strain \( C\Delta B739\_0832 \). (A) Growth curves of *Riemerella anatipestifer* CH-1, \( \Delta B739\_0832 \), and \( C\Delta B739\_0832 \) strains in Tryptic Soy Broth (TSB). Cells were inoculated in 25 ml of fresh TSB at 37°C with an initial OD\(_{600}\) of 0.05. OD\(_{600}\) values for each culture were subsequently measured every 2 h for 18 h. Data represent the mean values of three experiments. (B) Gene transcription levels in \( \Delta B739\_0832 \) strain were analyzed using quantitative PCR (qPCR). Transcription levels of \( B739\_0832 \) and the flanking genes \( B739\_0831 \) and \( B739\_0833 \) in *R. anatipestifer* CH-1 and \( \Delta B739\_0832 \) strains were measured. Expression of \( B739\_0832 \) was completely inactivated in the \( \Delta B739\_0832 \) mutant strain. Expression of upstream gene \( B739\_0831 \) and downstream gene \( B739\_0833 \) had no significant difference compared to wild type. Data were analyzed using Student’s t-test. Error bars represent standard deviations of three independent repeats.

**FIGURE 2** | Membrane localization of *R. anatipestifer* CH-1 \( B739\_0832 \) protein. Isolated subcellular fractions of *R. anatipestifer* cells were prepared as described in “Materials and Methods” and analyzed using Western blot. Results indicate that \( B739\_0832 \) protein localizes in the outer membrane. OmpA is a confirmed outer membrane protein in *R. anatipestifer*, RecA is a known cytoplasmic protein and TonB is a known inner membrane protein. See Supplementary Figure 3 in the Supplementary Material for more information.

**FIGURE 3** | Survival rate in ducklings infected by *R. anatipestifer* CH-1, \( \Delta B739\_0832 \), or \( C\Delta B739\_0832 \) strains. Each group has 10 3-day-old ducklings, which was injected intramuscularly at a dose of \( 10^{10} \text{ CFU} \) to assess the survival rate. The group shown in red was injected with wild-type *R. anatipestifer* CH-1. The orange group was infected with \( \Delta B739\_0832 \). The green was injected with \( C\Delta B739\_0832 \). Blue represents the control injected with phosphate-buffered saline (PBS). Data were analyzed using a Log-rank (Mantel–Cox) test. Three stars indicate significant difference (\( p < 0.001 \)).
invasion tests were performed under similar testing conditions. After killing all extracellular bacteria by gentamicin, bacterial counts inside host cells infected by ΔB739_0832 strain were 1.36 ± 0.1 × 10³ CFU/well, which was approximately twofold lower than those infected by wild type (2.44 ± 0.2 × 10³; Figure 4B). The complemented strain CAB739_0832 had almost identical activities as wild type for both adhesion and invasion tests.

**Deletion of B739_0832 Gene Attenuated *Riemerella anatipestifer* Virulence**

To further evaluate the influence of ΔB739_0832 on systemic infection in vivo, bacterial loads in blood, liver, spleen, and brain from ducks infected by wild type, ΔB739_0832, or CAB739_0832 were quantified. Bacterial loads from all three groups were almost identical for the first 24 h after infection, with the exception of bacterial loads in liver. However, a difference slowly developed at 36 and 48 h (Figures 5A–D). In brain and blood, the difference developed at 36 h, earlier than in spleen, which did not show significant difference until 48 h, whereas, in liver, the bacterial loads were different since 12 h post-infection, and the difference grew more significant at 48 h (Figure 5C).

To further examine the effects of deletion of B739_0832 gene on virulence, we compared organ tissue lesions in ducks infected by wild-type, ΔB739_0832, or CAB739_0832 strains. At 36 h post-infection, we collected heart, liver, spleen, and brain tissue samples from groups of ducklings infected by each of the three strains for histopathological examination. The tissue samples were stained with hematoxylin and eosin to visualize lesions caused by *R. anatipestifer* invasion. All brain tissues exhibited no visible damage, suggesting that *R. anatipestifer* had not passed the blood–brain barrier yet at this time point. However, liver cord disorders, involving a large number of vacuole-like changes, were clearly visible in samples infected by wild-type and complementary strain CAB739_0832. Myocardial necrosis was also present in heart samples infected by wild-type and CAB739_0832 strains. However, there were no visible lesions in liver or heart samples infected by the ΔB739_0832 strain (Figure 6).

**DISCUSSION**

*Riemerella anatipestifer* can infect a variety of domestic and wild birds, such as ducks, geese, and turkeys. Therefore, it is important to understand the pathogenic mechanisms of *R. anatipestifer* for controlling its spread. It is well known that outer membrane proteins in Gram-negative bacteria play important roles in stimulation of host immune systems (Navidinia et al., 2019).

OmpH has been demonstrated to be a major outer membrane protein present in *P. multocida* envelope (Lugtenberg et al., 1986). A study has shown that purified native OmpH protein from the *P. multocida* A3 strain could be used to elicit immune responses providing homologous protection in chickens (Luo et al., 1997), supporting OmpH in *P. multocida* as an exposed outer membrane protein. However, OmpH in *E. coli* and *S. typhimurium*, also known as Skp, has been identified as a periplasmic chaperone protein, despite initial reports of it as DNA binding protein (Holck et al., 1987), or outer membrane protein (Koski et al., 1989).

Our data suggest that B739_0832 protein in *R. anatipestifer*, a putative OmpH protein, is probably an outer membrane protein, consistent with it being more closely related to OmpH in *P. multocida* than in *E. coli* or *S. typhimurium*. The localization data suggest that B739_0832 protein is probably outer membrane protein, or at least outer membrane associated due to the limitation of our methods (Figure 2). Bioinformatics analysis found that over one-third amino acids in B739_0832 protein are hydrophobic, further suggesting that B739_0832 protein could
be a membrane-inserted protein. The impacts of $B739\_0832$ gene deletion on $R.\ anatipestifer$ virulence illustrated in this study, combining our previous study in successfully developing $B739\_0832$ protein-based ELISA method for detecting live $R.\ anatipestifer$ cells, suggest that $B739\_0832$ in $R.\ anatipestifer$ is probably an outer membrane protein, or at least is a membrane-associated protein.

Studies have shown that OmpH proteins in both $P.\ multocida$ and $E.\ coli$ can associate with lipopolysaccharide (LPS; Luo et al., 1997; Bulieris et al., 2003). LPS, as a cell wall component characteristic of Gram-negative bacteria, is a pathogen-associated molecule that plays an important role in triggering bacteria-infected host innate immune responses. Luo et al. believed that it was OmpH protein, not the small amount LPS contaminant, that elicited immune response in turkeys, whereas, in $E.\ coli$, Skp protein is more involved in facilitating outer membrane proteins through the periplasmic region. Skp’s association with LPS may partly be due to the numerous positively charged amino residues distributed throughout the protein and probably has no direct role as an antigen. In this study, we found that $B739\_0832$ affects $R.\ anatipestifer$ virulence and is likely involved in host cell attachment and invasion. It is very possible, due to its similarity to OmpH proteins in $P.\ multocida$ and $E.\ coli$, that $B739\_0832$ in $R.\ anatipestifer$ is also associated with LPS. However, it is not clear what role, if any, LPS plays in the virulence effects of $B739\_0832$. The relationship and association between $B739\_0832$ and LPS need to be studied further and will probably shed light on pathogenesis mechanisms of $R.\ anatipestifer$.

Our results show that $B739\_0832$, although not required for growth, is heavily involved in $R.\ anatipestifer$ virulence. Deletion of $B739\_0832$ gene greatly reduced its virulence: effecting lower mortality rate, higher survival rate in ducklings infected by $\Delta B739\_0832$, and much higher LD$_{50}$. $\Delta B739\_0832$ also showed attenuated pathogenic effects on host organs (no visible lesions found). However, only 50%
of ducklings infected by ΔB739_0832 survived for over 10 days, suggesting that B739_0832, although important for virulence, is not critical for pathogenesis. In fact, ΔB739_0832 could still attach and invade DEF cells and was detected in all examined host organs, albeit at lower amount than wild type. These lines of evidence strongly suggest that B739_0832 is involved in the initial invasion process of *R. anatipestifer*. It could be part of the host cell attachment process, which further confirms that B739_0832 is an outer surface protein.

The striking differences between B739_0832 and *E. coli* Skp protein are underscored in their sequence differences. Charged residues, especially positively charged residues, in *E. coli* Skp have been shown to play important functional roles (Bulieris et al., 2003). However, B739_0832 has fairly different distribution of charged residues than *E. coli* Skp. For example, comparing residues 21–45, about one-third (eight residues) of the total 25 residues changed from neutral to charged residues in B739_0832. This type of asymmetric distribution of charged residues is often observed in outer membrane proteins (Slusky and Dunbrack, 2013). It appears that these two proteins diverged at some point during evolution: one became an outer membrane protein, whereas another became a chaperone for outer membrane protein. However, it is not clear which role was earlier in the evolution process.

In summary, our earlier study showed that B739_0832-based ELISA could be used to detect *R. anatipestifer* of different serotypes with high sensitivity. In this study, we provide further evidence that B739_0832 is an outer membrane protein. We also demonstrated, for the first time, that B739_0832 is involved in *R. anatipestifer* virulence. B739_0832 is highly conserved among different serotypes. Its involvement in virulence further supports that B739_0832 is a good candidate as a universal antigen for developing vaccine for all serotypes.
DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Sichuan Agricultural University (Approval No. SCCXTD-2020-18).

AUTHOR CONTRIBUTIONS

QG and AC conceived and designed the experiments and wrote the manuscript. QG and SL performed the experiments. QG, AC, MW, RJ, SC, DZ, ML, XZ, QY, YW, SZ, JH, SM, XO, DS, and BT developed the methods. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fmicb.2021.708225/full#supplementary-material

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