Veterinary Microbiology 231 (2019) 93–99

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Riemerella anatipestifer gene *AS87_08785* encodes a functional component, GldK, of the type IX secretion system

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ARTICLE INFO

Keywords:
Riemerella anatipestifer
GldK
Gliding motility
Type IX secretion system
Bacterial virulence

ABSTRACT

Riemerella anatipestifer is an important pathogen of waterfowl, causing septicemic and exudative diseases. In our previous study, we demonstrated that the deletion of the *AS87_08785* gene significantly reduced the virulence of *R. anatipestifer* strain Yb2, but the mechanism remained unclear. In this study, *R. anatipestifer* strains with mutated or complemented *AS87_08785* genes were constructed and characterized. A sequence analysis indicated that the *AS87_08785* gene encoded a putative GldK protein, which localized to the membrane fraction in a western blotting analysis. The mutant strain Yb2ΔgldK displayed defective gliding motility on agar plates, reduced protease activity, and a reduced capacity for protein secretion. RNA sequencing and quantitative PCR analyses indicated that the transcription of 13 genes was downregulated in mutant Yb2ΔgldK. Animal experiments showed that the bacterial loads in the blood of Yb2ΔgldK-infected ducks were significantly reduced relative to those in wild-type strain Yb2 infected ducks. Most of the defective biological properties of the mutant were restored in complementation strain cYb2ΔgldK. Our results demonstrated that *R. anatipestifer* gene *AS87_08785* encoded a component of the type IX secretion system, GldK, which functioned in bacterial gliding motility, protein secretion, and bacterial virulence.

1. Introduction

Riemerella anatipestifer is a Gram-negative, non-spore-forming, rod-shaped, capsulated bacterium with gliding motility, belonging to the family Flavobacteriaceae in the phylum Bacteroidetes (Segers et al., 1993). It has a worldwide distribution, and causes acute or chronic septicemia, fibrous pericarditis, airsacculitis, perihepatitis, caseous salpingitis, and vegetative disorders. Infection with *R. anatipestifer* predominantly causes disease in domestic ducks, and is responsible for major economic losses in the poultry industry (Segers et al., 1993). Twenty-one serotypes have been identified with agglutination tests, but no significant cross-protection has been identified (Pathanasophon et al., 2002). Several virulence factors, including VapD, CAMP cohemolysin, and lipopolysaccharide-synthesis-associated genes, have been reported (Chang et al., 1998; Crasta et al., 2002). Recently, 49 virulence genes have been identified in *R. anatipestifer* strain Yb2 using random transposon mutagenesis (Wang et al., 2015). The virulence of the mutant strain RA256, in which the *AS87_08785* gene was deleted, was reduced over 7,000-fold, but the mechanism remains unknown.

The novel protein secretion system known as the type IX secretion system (T9SS) or Por secretion system, which is associated with gliding motility and the secretion of virulence factors, has recently been identified in *R. anatipestifer* (McBride and Zhu, 2013). Motility plays a major role in the pathogenesis in many members of the phylum Bacteroidetes. T9SS and bacterial gliding motility are also associated with the secretion of proteins that are thought to be virulence factors in many pathogens (Sato et al., 2013; Shrivastava et al., 2012). Genetic analyses have shown that the core of the T9SS gene cluster includes *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, *sprT*, *sov*, *porW*, *porU*, and *porV* (Kharade and McBride, 2015; Shrivastava et al., 2013). Proteins secreted by T9SS have predicted N-terminal type 1 signal peptides, which allow their transit across the cytoplasmic membrane via the general secretion system. They also have conserved C-terminal domains (CTDs) that appear to direct them to the T9SS for secretion across the outer membrane (Shrivastava et al., 2012; Slakeski et al., 2011). *sprT*, which encodes a core protein of T9SS, is involved in proteins secretion in *R. anatipestifer* and contributes to its virulence by exporting key proteins (Guo et al., 2017). Bacteria with a functional motility system form spreading colonies on agar plates, which allows the easy identification of motility mutants (McBride and Zhu, 2013). In this study, we showed that the

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https://doi.org/10.1016/j.vetmic.2019.03.006
Received 24 November 2018; Received in revised form 6 March 2019; Accepted 7 March 2019

AS87_08785 gene encoded a putative gliding-motility protein, GldK, which was required for bacterial protein secretion, gliding motility, and virulence.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (approval no. Shvri-po-2017090876), and was conducted in strict accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals. One-day-old Cherry Valley ducks were obtained from the Zhuang Hang Duck Farm (Shanghai, China) and housed in cages under a controlled temperature of 28–30 °C under biosafety conditions, with water and food *ad libitu*m.

2.2. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids are listed in Supplementary Table S1. *Riemerella anatipestifer* Yb2 was the virulent wild-type strain used in this study. The mutant strain, Yb2ΔgldK, in which the *AS87_08785* gene was deleted, was constructed by the insertion of transposon Tn4351 (Wang et al., 2015). The complementation strain, cYb2ΔgldK, was constructed using plasmid pCP29, as described previously. The *R. anatipestifer* strains were grown at 37 °C in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA). The *Escherichia coli* strains were grown at 37 °C on Luria–Bertani (LB) plates or in LB broth. Antibiotics were added at the following concentrations: ampicillin (100 μg/mL), chloramphenicol (30 μg/mL), erythromycin (0.5 μg/mL), kanamycin (50 μg/mL), and cefoxitin (5 μg/mL).

2.3. Identification of mutant strain Yb2ΔgldK

The transposon Tn4351 was introduced into the *R. anatipestifer* wild-type strain Yb2 from *E. coli* BW19851 by conjugation, as described previously (Hu et al., 2012). The primer pairs RA 16S rRNA-F/RA 16S rRNA-R, specific for *R. anatipestifer* 16S rRNA, and Erm-F/Erm-R, specific for Tn4351, are described in Supplementary Table S1 and were used to identify the Tn4351 insertion in the *R. anatipestifer* mutant with PCR amplification.

A Southern blotting analysis confirmed one insertion of transposon Tn4351 in the Yb2 genome, as described previously (Wang et al., 2015). The site of transposon insertion in the mutant strain was determined to be at the putative *gldK* gene, using inverse PCR (Alvarez et al., 2006). Briefly, the chromosomal DNA of the mutant was digested with HindIII and ligated with T4 ligase, which resulted in the formation of closed circles. The Tn*4351*-specific primers TN-1 and IS4351-F were used to amplify the DNA adjacent to the insertion site using premix LA Taq (Takara, Dalian, China). The DNA sequence data were compared with a database using the basic local alignment search tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The mutant was designated Yb2ΔgldK.

2.4. Complementation of the mutant Yb2ΔgldK strain

Complementation of the mutant strain was achieved with the shuttle plasmid pCP29, which contained an expression cassette that included the *R. anatipestifer ompA* promoter (Hu et al., 2012). The *AS87_08785* open reading frame was amplified from wild-type strain Yb2 with the primers AS87_08785 comp-F/AS87_08785 comp-R (Supplementary Table S1). The PCR product and plasmid pCP29 (Wang et al., 2014a) were separately digested with SphI and XhoI, and then the PCR product (*AS87_08785*) was cloned into pCP29 to generate plasmid pCP29-AS87_08785 under the control of the *ompA* promoter, as described previously (Hu et al., 2012). The plasmid was first introduced into *E. coli* S17-1 by transformation. It was then transferred into the mutant strain by conjugation. Transformants were selected on TSA containing 5 μg/mL cefoxitin and 50 μg/mL kanamycin, and identified by PCR amplification with primers AS87_08785-comp-F/AS87_08785-comp-R and RA 16S rRNA-F/RA 16S rRNA-R. The complementation strain was designated cYb2ΔgldK.

2.5. Bacterial growth, adherence, and invasion assays

A growth curve was constructed as described previously (Hu et al., 2012). Briefly, the wild-type strain Yb2, mutant strain Yb2ΔgldK, and complementation strain cYb2ΔgldK were grown in TSB at 37 °C for 8 h, with shaking. Equal amounts of each bacterial culture were then transferred into fresh TSB in a ratio of 1:100 (vol/vol) and incubated at 37 °C, with shaking at 200 rpm. Bacterial growth was measured as described previously (Wang et al., 2015), by counting the numbers of bacterial colony-forming units (CFU) at 1 h intervals for 18 h.

Adhesion and invasion assays were performed in Vero cells (ATCC CCL-81), as described previously (Hu et al., 2012). Briefly, a 24-well tissue culture plate was seeded with 10^5 cells/well in Dulbecco's Modified Eagle's Medium (DMEM Biowest, France) containing 10% fetal bovine serum. After incubation at 37 °C under 5% CO_2 for 24 h, the cell monolayer was rinsed with phosphate-buffered saline (PBS) and infected with bacteria at a multiplicity of infection (MOI) of 100 by centrifugation at 100 × *g* for 5 min at room temperature. The infected cells were incubated at 37 °C under 5% CO_2 for 1.5 h, washed three times with sterile PBS, and lysed with 0.1% trypsin (100 μL/well). The cell suspensions were diluted 10-fold and plated onto TSA plates to determine the numbers of viable bacterial cells. For the invasion assay, the extracellular bacteria were killed by incubating the monolayer with DMEM supplemented with 100 μg/mL gentamycin for an additional 1 h. The bacteria were then incubated and washed three times with PBS. All these assays were performed in triplicate and replicated three times.

2.6. GldK expression, antibody production, and western blotting analysis

A 1,410-bp fragment of the *gldK* gene was amplified with primers GldK-segmental-pro-F/GldK-segmental-pro-R (Supplementary Table S1), digested with *Bam*HI and *Sal*I, and ligated into the pET30a vector to construct the recombinant plasmid pET-*gldK*. The construction of pET-*gldK* was confirmed with DNA sequencing. The expression of the histidine-tagged recombinant GldK protein (rGldK) was induced in *E. coli* strain BL21(DE3) cells by treatment with 1 mM isopropyl-β-D-1-thiogalactopy-ranoside (IPTG) for 6 h at 37 °C, with shaking. The cells were harvested with centrifugation at 10,000 × *g* for 5 min at 4 °C, resuspended in lysis buffer (20 mM Na_3PO_4, 0.5 M NaCl [pH 7.4]), lysed by sonication, and purified with HisTrap affinity columns (GE Healthcare, Uppsala, Sweden), as described in the manufacturer's protocol. The purified fractions were confirmed with SDS-PAGE. The protein concentrations were measured with a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China), with bovine serum albumin as the standard.

Antibodies were raised against rGldK, as previously described (Wang et al., 2016). Briefly, two New Zealand rabbits were injected subcutaneously with 1 mg of purified rGldk from *R. anatipestifer* in Montanide ISA 50 V adjuvant (Seppic, Paris, France). The injections were administered three times, at 3-week intervals. Blood samples were collected before injection and 2 weeks after the third injection, and the antibody titers against rGldK in the sera were determined with an enzyme-linked immunosorbent assay. The qualified antiserum was used for the western blotting analysis.

To identify the Yb2ΔgldK mutant and complementation strains, whole-protein extracts of wild-type strain Yb2 (positive control), mutant strain Yb2ΔgldK, and complementation strain cYb2ΔgldK were separated with SDS-PAGE and transferred to nitrocellulose membranes

(Millipore, Billerica, MA, USA). The membranes were blocked for 2 h at 37 °C with PBS containing 5% nonfat milk, washed with PBS containing 0.05% Tween 20, and then incubated for 2 h with rabbit anti-GldK antiserum. After incubation for 1 h, the membrane was washed with PBS containing 0.05% Tween 20, and then a horseradish-peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (Bio-Rad Laboratories, Hercules, CA, USA) was applied. The specific bands were developed with the Basic Luminol Chemiluminescent Kit (S-Wb001), visualized with the Tanon 5200 automated chemiluminescence image analysis system (Tanon, Shanghai, China), and quantified with the ImageJ software (National Institutes of Health, Rockville, USA). A rabbit anti-TonB antibody was used as the control for protein loading.

The subcellular localization of GldK was determined with a western blotting analysis. Briefly, the cytosolic and membrane fractions of wild-type strain Yb2 were prepared with a bacterial membrane protein extraction kit (BestBio, Shanghai, China), according to the manufacturer's protocol. The proteins were quantified with a BCA protein assay kit (Beyotime). The proteins were separated with SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blotting was performed as described above. A rabbit anti-TonB antibody was used to control for protein loading.

2.7. Microscopic observation of colony spreading

The wild-type strain Yb2, mutant strain Yb2ΔgldK, and complementation strain cYb2ΔgldK were grown on TSA plates for 12 h, washed with TSB, and diluted to 2.5×10^3 CFU/ml. Aliquots (50 μL) of the cultures were plated onto TSB medium containing 0.5% agar. After incubation at 37 °C for 24 h, the colonies of each strain were examined with phase-contrast microscopy *(Nikon D-Eclipse C1, Japan)*.

2.8. Measurement of protease activity

The proteolytic activity of the *gldK* gene product was assayed as described previously (Newton et al., 1997), with modifications. Briefly, *R. anatipestifer* wild-type strain Yb2, mutant strain Yb2ΔgldK, and complementation strain cYb2ΔgldK were grown on TSA plates for 12 h, washed with PBS, adjusted to 2.5×10^9 CFU/mL, and used to inoculate 5 mL of animal-derived component-free (ADCF)-MAb medium (Hyclone). The strains were cultured for 8 h culture at 37 °C, with shaking at 220 rpm. The cultures were then centrifuged at 19,950 × *g* for 10 min, and the residual cells were removed from the supernatants by filtering them through 0.45 μM *HT Tuffryn* syringe filters (Pall Life Sciences, Ann Arbor, MI). The bacterial pellets were dried at 80 °C in a heat block for 3 h, and the dry weights of the cell pellets were determined to calculate the proteolytic activity. The proteolytic assay was performed as follows. Azocasein (2%; Sigma-Aldrich) solution was prepared in 0.05 M Tris−HCl (pH 7.4). The cell-free supernatant (50 μL) was mixed with 50 μL of the azocasein substrate, and incubated at 37 °C for 3 h. Triplicate assays were performed for each supernatant sample and the negative controls (50 μL of ADCF-MAb medium). After incubation, 130 μL of 10% trichloroacetic acid was added to each sample, mixed, and incubated at room temperature for 10 min. The samples were centrifuged at 19,950 × *g* for 20 min to remove the precipitated azocasein. An aliquot (100 μL) of the soluble fraction of each sample was added to a 96-well plate, and 200 μL of 1 M NaOH was added to each well and mixed. The optical density at a wavelength of 450 nm (OD_{450}) was determined with an iMark Microplate Absorbance Reader (Bio-Rad). For each supernatant sample, the raw OD_{450} values for triplicate assays were averaged, and the mean negative control OD_{450} value was subtracted from these values. The proteolytic activity per mg of dry cells was calculated as (mean sample OD_{450} - mean negative control OD_{450}) × 1000 × 100/dry weight of the bacterial pellet.

2.9. Liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis of protein secretion

Riemerella anatipestifer wild-type strain Yb2 and mutant strain Yb2ΔgldK were grown in 200 mL of ADCF-MAb medium at 37 °C with shaking until the OD_{600} reached 0.8 (almost 8 h), and were then centrifuged at 8000 × *g* for 10 min. The supernatants were purified by passage through 0.22 μm PVDF filters. The supernatant of each strain was concentrated to 2 mL with a 3 K Amicon Ultra Centrifugal Filter Unit (Sigma-Aldrich) and stored at -80 °C until required. Protein quality was determined with SDS-PAGE followed by Coomassie Brilliant Blue staining to visualize the presence of protein bands. The qualified proteins were analyzed with LC–MS/MS, as described previously (Kharade and McBride, 2015). The MS/MS data were screened against the UniProt database with the MASCOT engine. The BLAST server was used to identify sequences homologous to the identified proteins of *R. anatipestifer* Yb2 and their putative functions.

2.10. RNA sequencing and differential expression analysis

Total RNA was isolated from wild-type strain Yb2 and mutant strain Yb2ΔgldK with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the rRNA was removed with the Ribo-Zero Magnetic Gold Kit (Epicenter, USA), according to the manufacturer's instructions. Illumina RNA-Seq libraries were generated and sequenced for 100 cycles on the Illumina HiSeq 2000 Sequencing System, according to the manufacturer's instructions (Wang et al., 2016). Low quality reads and adaptors were removed from the raw reads. The cleaned reads were aligned to the *R. anatipestifer* Yb2 genome with the RNASequel software HISAT (version 2.0.1-beta) (Wilson and Stein, 2015). The trimmed reads were aligned to the *R. anatipestifer* Yb2 genome using the TopHat2 software (version 2.0.9) (Kim et al., 2013). The transcript levels were calculated as fragments per kilobase cDNA per million fragments mapped (FPKM). Differentially expressed genes and their fold changes (cutoff = 2.0) were analyzed with the Cufflinks software (version 2.1.1) (Trapnell et al., 2010), and were considered statistically significant if the fold change was > 2.0 and the p value < 0.05.

2.11. Real-time quantitative PCR (qPCR) analysis

Realtime quantitative PCR was performed to confirm the transcription levels of the differentially expressed genes identified in the RNA-Seq analysis. Gene-specific primers were designed with the Primer3 online software version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). The gene encoding L-lactate dehydrogenase (*ldh*) was amplified with primers RA ldh-F/RA ldh-R (Supplementary Table S1) as the internal control. Total RNA was isolated from wild-type strain Yb2 and mutant strain Yb2ΔgldK with TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. All RNA samples were treated with the TURBO DNA-free Kit (Ambion, Grand Island, NY, USA) to remove DNA contamination. cDNA was synthesized with PrimeScript RT Master Mix (Takara). Real-time qPCR was performed in GoTaq qPCR Master Mix (Promega, Fitchburg, WI, USA), with the following parameters: 95 °C for 1 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s; followed by one cycle of 60 °C for 15 s and 95 °C for 15 s. The reactions were performed in triplicate and run on the Mastercycler ep realplex4 apparatus (Eppendorf, Germany). The transcription levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Dou et al., 2017).

2.12. Assessment of virulence in vivo

The influence of the *gldK* gene deletion on the virulence of *R. anatipestifer* was determined with a previously described protocol (Wang et al., 2015). Briefly, 30 18-day-old Cherry Valley ducks were divided randomly into three groups and infected intramuscularly at a dose of 10^6 CFU with the wild-type strain Yb2, mutant strain Yb2ΔgldK, or

complementation strain cYb2ΔgldK in 0.5 mL of PBS. To avoid the potential conversion of the strains, Yb2, Yb2ΔgldK and cYb2ΔgldK were characterized by PCR analysis before infection. Blood samples were collected at 3, 6, 12, and 24 h post infection, diluted appropriately, and plated on TSA for bacterial counting (Wang et al., 2016). Recovered bacteria from blood culture were subjected to PCR analysis to confirm the bacterial strain.

2.13. Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Adhesion and invasion data, bacterial loads in the blood of ducks, and qPCR data were two tailed, and a p value of < 0.05 was considered significant. Multiple-group comparisons were made with ANOVA.

3. Results

3.1. Bioinformatic analysis of the R. anatipestifer gldK gene

Sequence and local BLAST analyses revealed that the transposon Tn4321 was inserted at the predicted *gldK* gene in the *R. anatipestifer* genome, and was 1,410-bp in length and encoded the predicted 470-amino-acid GldK. A similarity search of the nucleotide databases at the National Center for Biotechnology Information using the BLAST program showed that the *gldK* gene was highly conserved in *R. anatipestifer* strains, sharing 100% identity in *R. anatipestifer* strains 153, 17, RA-CH-2 and RA-GD, and 94%–97% identity in *R. anatipestifer* strains RA-CH-1, DSM 15868, ATCC 11845, NCTC 11014, CH3, and HXb2. GldK is a component of T9SS and was originally identified as a protein required for bacterial gliding motility (Braun et al., 2005; Nelson et al., 2007). It is also assumed to be involved in protein secretion (Saiki and Konishi, 2007; Sato et al., 2010).

3.2. Identification of mutant strain Yb2ΔgldK and complementation strain cYb2ΔgldK

To demonstrate that the *gldK* gene was defective in the mutant strain, PCR amplification was performed with primers AS87_08785 comp-F/AS87_08785 comp-R. No 1410-bp fragment of *gldK* was amplified from mutant stain Yb2ΔgldK (Fig. 1A, lane 1), but the 1410-bp fragment of *gldK* was amplified from wild-type strain Yb2 (Fig. 1A, lane 2) and complementation strain cYb2ΔgldK (Fig. 1A, lane 3). PCR with primers RA 16S rRNA F/RA 16S rRNA R and Erm-F/Erm-R amplified a 792-bp fragment of 16S rRNA and a 644-bp fragment of Erm, respectively, from mutant strain Yb2ΔgldK (Fig. 1A, lane 5), whereas the Erm gene was not amplified from wild-type strain Yb2 (Fig. 1A, lane 6). The correct fragments of both 16S rRNA and Erm were amplified from complementation strain cYb2ΔgldK (Fig. 1A, lane 7). A western blotting analysis with a rabbit anti-GldK polyclonal antibody detected a 53.5-kDa protein, which was consistent with the estimated size of the GldK protein, in whole protein extracts of wild-type strain Yb2 (Fig. 1B, lane 1), but not in mutant strain Yb2ΔgldK (Fig. 1B, lane 2). Complementation strain cYb2ΔgldK displayed the 53.5-kDa band (Fig. 1B, lane 3).

3.3. Deletion of gldK gene reduced bacterial adherence and invasion capacities

When bacterial growth was examined in TSB, no significant differences were detected among wild-type strain Yb2, mutant strain Yb2ΔgldK, and complementation strain cYb2ΔgldK (Fig. 1C).

The adherence and invasion capacities of Yb2, Yb2ΔgldK, and cYb2ΔgldK were assessed in Vero cells. Both the adherence and invasion capacities of mutant strain Yb2ΔgldK were significantly reduced compared with those of Yb2 ($p < 0.05$). These capacities were not restored to the complementation strain cYb2ΔgldK (Fig. 1D, 1E).

3.4. Subcellular localization of GldK

To determine the subcellular localization of GldK in *R. anatipestifer* Yb2, the cytoplasmic and membrane fractions were extracted with a bacterial membrane protein extraction kit (BestBio), according to the manufacturer's protocol. A western blotting analysis detected a single 53.5-kDa band corresponding to GldK in the whole-cell proteins (Fig. 1F, lane 1) and purified membrane fraction (Fig. 1F, lane 3). No GldK band was detected in the cytoplasmic protein fraction, indicating that GldK was expressed in the cytomembrane of *R. anatipestifer* (Fig. 1F, lane 2). TonB was used as the loading control.

3.5. gldK deletion mutant is defective in gliding motility

The motility phenotypes of wild-type strain Yb2, mutant strain Yb2ΔgldK, and complementation strain cYb2ΔgldK were examined with time-lapse microscopy. The Yb2 cells formed spreading colony (Fig. 2A), whereas the Yb2ΔgldK cells formed no spreading colony on agar medium (Fig. 2B), suggesting that the mutant strain was defective in gliding motility. Gliding motility was restored in complementation strain cYb2ΔgldK, which formed spreading colonies (Fig. 2C).

3.6. Deletion of the gldK gene significantly attenuates bacterial virulence

To assess the role of *gldK* in the systemic invasion and dissemination of *R. anatipestifer*, the bacterial loads in the blood of ducks infected with wild-type strain Yb2, mutant strain Yb2ΔgldK, or complementation strain cYb2ΔgldK were determined. Significantly fewer bacteria were recovered from the blood of ducks infected with strain Yb2ΔgldK compared with the blood of ducks infected with wild-type strain Yb2, at 3, 6, 12 and 24 h postinfection. The bacterial loads in the blood of complementation strain cYb2ΔgldK infected ducks were similar to those in the Yb2 infected ducks at 24 h post infection (Fig. 3). Recovered bacteria from each blood culture were confirmed by PCR analysis as described in 3.2 section, no reversion of the output strain was found.

3.7. gldK deletion mutant displays defective protein secretion

The cell-free culture fluids of wild-type strain Yb2 and mutant strain Yb2ΔgldK were collected, and subjected to an SDS-PAGE analysis to determine the differential secretion of proteins in these strains (Fig. 4A). The proteins in the cell-free culture fluid from the two strains were then identified with LC–MS/MS. Four proteins, which had typical T9SS CTDs, were absent from the Yb2ΔgldK culture (Table 1). A further assay revealed that mutant strain Yb2ΔgldK was defective in the digestion of proteins, supporting the notion that enzymes involved in the digestion of these polymers are secreted by T9SS. The proteolytic activity of complementation strain cYb2ΔgldK was restored (Fig. 4B).

3.8. gldK disruption affects gene expression

A strand-specific Illumina RNA-Seq analysis indicated that 22 genes were differentially expressed in mutant strain Yb2ΔgldK and wild-type strain Yb2. qPCR confirmed that 13 genes were significantly down-regulated (over twofold) at the transcription level (Table 2). Genes *AS87_RS08465*, *AS87_RS01370*, *AS87_RS01360*, *AS87_RS01355*, *AS87_RS01350*, *AS87_RS07855*, and *AS87_RS08825* encoded gliding motility protein GldM, a membrane protein, hemolysin D, a transporter, a heavy metal efflux pump, CzcA family member, ArsR family transcriptional regulator, and 1-aminocyclopropane-1-carboxylate deaminase, respectively. The other six genes *AS87_RS01190*, *AS87_RS01365*, *AS87_RS09460*, *AS87_RS02680*, *AS87_RS09445*, and *AS87_RS05240* encode hypothetical proteins. *AS87_RS08465* encoded gliding motility protein GldM, a component of T9SS, which is involved in protein

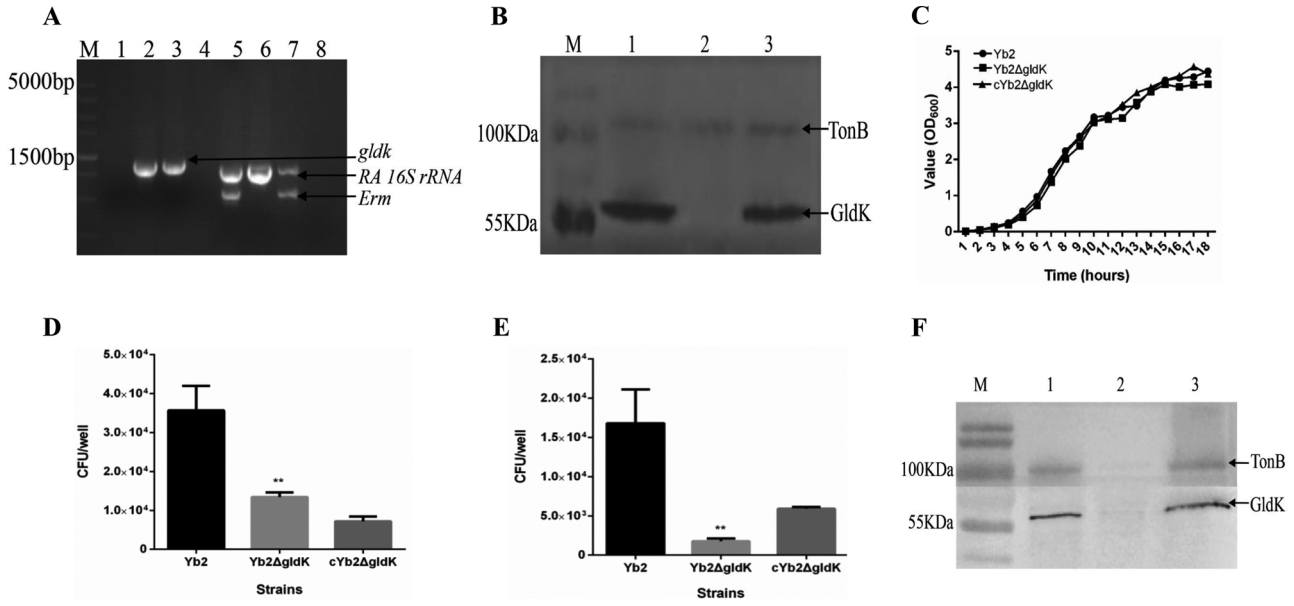

Fig. 1. Characterization of mutant strain Yb2ΔgldK and complementation strain cYb2ΔgldK. (A) PCR amplification. M: Takara DM2000 marker (CWBIO, Beijing China). Lanes 1–4, products were amplified using primer pairs AS87_08785 comp-F/AS87_08785 comp-R; Lanes 5–8, products were amplified using primer pairs RA 16S rRNA F/RA 16S rRNA R and Erm-F/Erm-R. Lanes 1 and 5, mutant strain Yb2ΔgldK; lanes 2 and 6, wild-type strain Yb2; lanes 3 and 7, complementation strain cYb2ΔgldK; lanes 4 and 8, negative control. No 1413-bp fragment of *gldK* was amplified from mutant strain Yb2ΔgldK (lane 1); no 644-bp fragment of Erm was amplified from wild-type strain Yb2 (lane 6). (B) Western blotting analysis. Cytoplasmic and membrane fractions were extracted and analyzed with western blotting. Lane 1, whole cell proteins from wild-type strain Yb2; lane 2, cytoplasmic protein extract from Yb2; lane 3, membrane protein extract from Yb2. TonB was used as the loading control. (C) Growth curves in TSB culture at 37 °C with 220 rpm shaking. OD_{600} values were measured at 1 h intervals. The experiment was repeated three times, and data presented are means. Error bars represent standard deviations. (D) Bacterial adherence assay. (E) Bacterial invasion assay. Adherence and invasion assays were performed in Vero cells (ATCC CCL-81) with an MOI of 50. Data represent counts of bacteria bound to or that have invaded Vero cells in each well of a 24-well plate. Error bars represent means ± standard deviations of three independent experiments ($^{**}p < 0.01$). (F) Localization of GldK. Cytoplasmic and membrane fractions were extracted with a bacterial membrane protein extraction kit (BestBio, Shanghai, China) and analyzed with western blotting. Lane 1, whole cell proteins from wild-type strain Yb2; lane 2, cytoplasmic protein extract from Yb2, no GldK protein was shown; lane 3, membrane protein extract from Yb2, indicating that GldK is a membrane protein. TonB was used as the loading control.

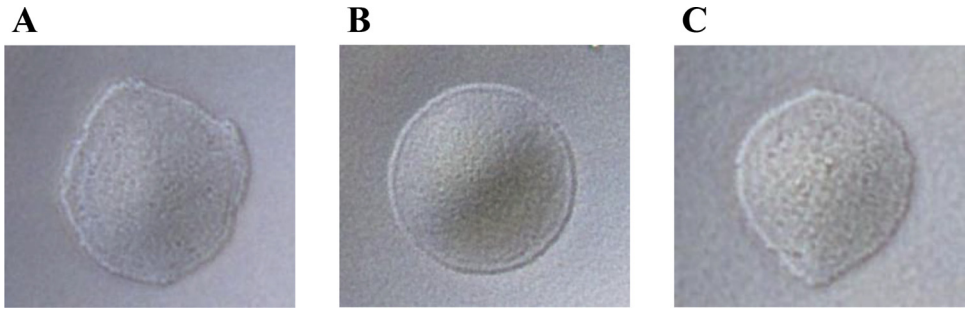

Fig. 2. Observation of bacterial motility. Bacterial strains grown from single cells on TSA agar plates at 37 °C for 24 h. Photomicrographs were taken with a Photometric Cool SNAP cf2 camera mounted on an Olympus IMT-2 phase-contrast microscope. (A) Wild-type strain Yb2, colony has an irregular edge due to bacterial motility. (B) Mutant strain Yb2ΔgldK, the colony has a neat edge due to loss of motility. (C) Complementation strain cYb2ΔgldK, the colony is restored to irregular edge.

secretion and cell movement. These results suggested that AS87_RS08785 regulated genes mainly responsible for bacterial protein secretion and gliding motility in *R. anatipestifer*.

4. Discussion

Protein secretion systems are vital for prokaryotic life because they allow bacteria to acquire nutrients, communicate with other species, defend themselves against biological and chemical agents, and facilitate disease through the delivery of virulence factors (Lasica et al., 2017). In this study, we characterized the *AS87_RS08785* mutant strain Yb2ΔgldK and found that the *AS87_RS08785* gene was associated with bacterial gliding motility, virulence, and protein secretion. This suggested that GldK was a component of T9SS, a recently discovered novel secretion system in some species of the phylum Bacteroidetes, including *Flavobacterium johnsoniae*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *R. anatipestifer* (McBride and Zhu, 2013).

Numerous studies have shown that T9SS is involved in the secretion of virulence factors. For instance, in *P. gingivalis*, it secretes extracellular and cell-surface gingipain proteases, which are major virulence factors involved in periodontal pathogenesis (Curtis et al., 2001). *Flavobacterium columnare* T9SS secretes the enzyme chondroitin sulfate lyase, which is associated with the pathogen's virulence (Li et al., 2017). The role of T9SS is to ensure cell survival and fitness in response to the microorganism's habitat by transporting necessary proteins and other molecules, and in its virulence, nutrition, and movement (gliding motility). The variety of secreted proteins is large, even within a single species, and they include numerous adhesins and hydrolytic enzymes used for the attachment and degradation of large organic compounds, such as proteins, cellulose, and chitin (Lasica et al., 2017). Twelve *gld* genes (*gldA*, *gldB*, *gldD*, *gldF*, *gldG*, *gldH*, *gldI*, *gldJ*, *gldK*, *gldL*, *gldM*, and *gldN*) have been identified, which play important roles in gliding motility (McBride and Zhu, 2013). Disruption any of these genes results in the complete loss of gliding motility, a deficiency in chitin utilization, and resistance to bacteriophages that infect wild-type cells (Agarwal et al., 1997; Braun and McBride, 2005; Sato et al., 2010). *gldK*, *gldL*,

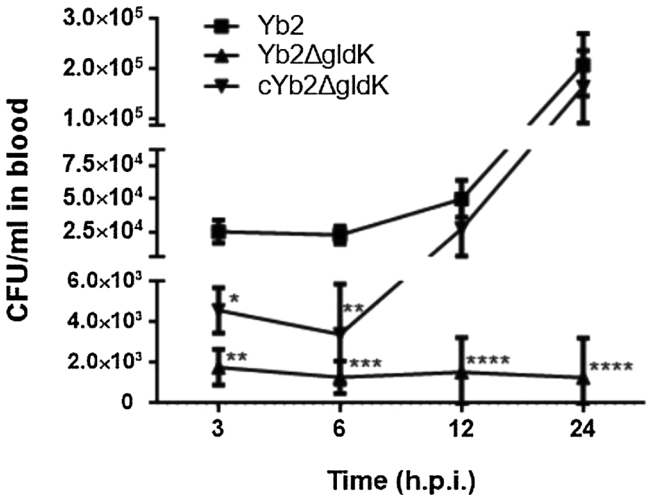

Fig. 3. Virulence determination. Ducks were infected with 10^6 CFU of PCR confirmed wild-type strain Yb2, mutant strain Yb2ΔgldK, or complementation strain cYb2ΔgldK. Blood samples were collected for bacterial load determination at 3, 6, 12, and 24 h post infection. Bacterial CFUs were counted and analyzed by PCR to confirm the strain. Data are presented as the means ± standard deviations of 10 infected ducks. Asterisks indicate statistically significant difference between groups ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

gldM, and *gldN* were originally identified as the genes responsible for gliding motility in *F. johnsoniae* (Braun et al., 2005). Later, orthologues of these genes (*porK*, *porL*, *porM*, and *porN*, respectively) were shown to be required for the secretion of *P. gingivalis* gingipains (Sato et al., 2010). GldK is a lipoprotein linked to the rapid surface gliding motility found in certain Bacteroidetes, including *Cytophaga johnsonae* (*F. johnsoniae*) (Braun et al., 2005), and it is homologous to GldJ (Braun and McBride, 2005). Our results confirmed that in *R. anatipestifer*, GldK was a membrane-linked protein and the mutant strain Yb2ΔgldK was defective in gliding motility.

Species in the phylum Bacteroidetes, including *F. columnare*, *F. johnsoniae*, *P. gingivalis*, *T. forsythia*, and *R. anatipestifer*, contain T9SSs that play central roles in the pathogenesis of these species (Penttinen et al., 2018). This was confirmed in our study by T9SS-*gldK* mutant strain Yb2ΔgldK, which showed reduced virulence.

A genomic analysis demonstrated that *R. anatipestifer* encoded several genes involved in gliding motility, including gldA, gldB, gldC, gldD, gldF, gldH, gldJ, gldK, gldL, gldM, gldN, porP, and porT (Abby et al., 2016). Many of the proteins encoded by these genes are predicted to localize to the cellular envelope. In F. johnsoniae, gldK, gldL, gldM, and gldN cluster together in two adjacent operons, although gldK is transcribed separately from the other three genes (Braun et al., 2005). A similar arrangement is found in *R. anatipestifer* and other Bacteroidetes, such as F. psychrophilum and *C. hutchinsonii* (McBride et al., 2009). This organization suggested that the protein products of these genes acted together as parts of a complex, and the extensive conservation of the genes encoding this protein secretion system also indicated that it also probably functioned in *R. anatipestifer*. Analysis of this system in *R. anatipestifer* should provide insight into the pathogenesis of the diseases it causes.

A previous study reported that the *sprT* gene encodes the T9SS component of SprT, which is involved in protein secretion and virulence in *R. anatipestifer* (Guo et al., 2017). The other functional components of T9SS in *R. anatipestifer* are unclear. In this study, we investigated the deletion of the *R. anatipestifer* Yb2 gene *gldK*, which encoded a component of T9SS. This attenuated the virulence of mutant strain Yb2ΔgldK more than 7,000-fold and significantly affected its adherence and invasiveness. The bacterial loads in the blood of ducks infected with Yb2ΔgldK were significantly reduced relative to those in ducks infected with wild-type strain Yb2 or complementation strain cYb2ΔgldK. These findings clearly showed that T9SS played an important role in the pathogenicity of *R. anatipestifer*. An SDS-PAGE analysis of cell-free cultures revealed that many secreted proteins were reduced or absent after *gldK* mutation, and a proteolytic activity assay indicated that some of these proteins were proteases. Our LC–MS/MS analysis revealed that four proteins with typical T9SS CTDs were absent from mutant strain Yb2ΔgldK. A further assay demonstrated that mutant strain Yb2ΔgldK was defective in the digestion of proteins, supporting the notion that enzymes involved in the digestion of these polymers were secreted by T9SS. All the CTDs belonged to the TIGRFAM protein domain family TIGR04183 (which are referred to as "type A CTDs"), and the T9SS components GldK and SprT may complemented each other in function (Li et al., 2017).

In conclusion, we characterized the *R. anatipestifer AS87_08785* gene, encoding the T9SS component GldK, which was responsible for bacterial protein secretion, gliding motility, and virulence. Further studies of T9SS in *R. anatipestifer* are required to better understand the pathogenic mechanism of this bacterium and to develop an effective strategy for its control.

Conflict of interest

The authors declare that they have no competing interests.

Funding

This work was supported by National Key R&D Program (2016YFD0500805), the Shanghai Key Project on Agricultural Development through Science and Technology (2016HNG4-1) and Co-innovation of Science and Technology Innovation Project in Chinese

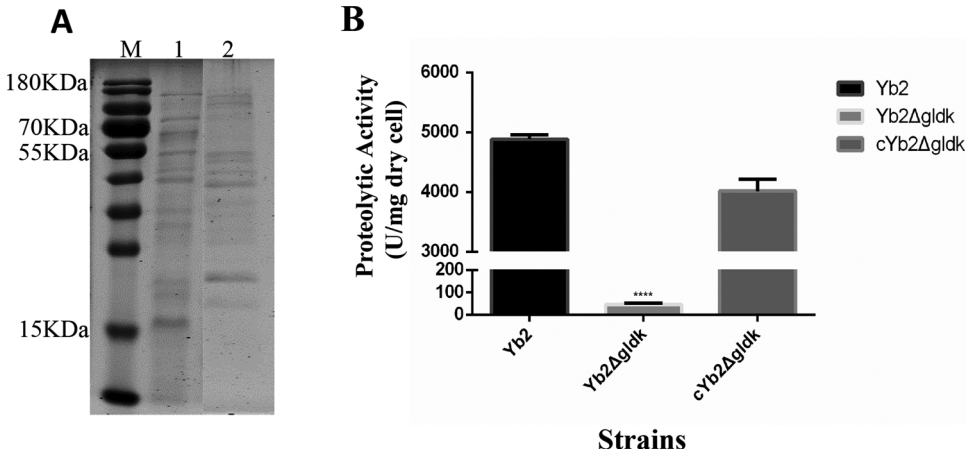

Fig. 4. Determination of protein secretion and proteolytic activity. (A) SDS-PAGE analysis of secreted proteins. Lane 1: cell-free culture fluid of wild-type strain Yb2; lane 2: cell-free culture fluid of mutant strain Yb2ΔgldK. (B) Protease activities of wild-type strain Yb2, mutant strain Yb2ΔgldK, and complementation strain cYb2ΔgldK. $^{****}p < 0.001$.

Table 1
Identification of differentially expressed proteins with T9SS CTDs in *R. anatipestifer* wild-type strain Yb2 and mutant strain Yb2ΔgldK[a].

Locus tag	Molecular mass (KDa)	T9SS CTD[b]	Proteins	Predicted protein function	Relative change of protein[c]
AS87_RS03090	13.49	+	Uncharacterized protein	Unknown	6.1854
AS87_RS02625	39.812	+	Endonuclease i	Endonuclease activity	0.2762
AS87_RS00835	161.5	+	Peptidase s8 and s53 subtilisin kexin sedolisin	Serine-type endopeptidase activity	0.1309
AS87-RS00980	68.225	+	Metallophosphoesterase (MPPE)	Acid phosphatase activity	0.2494

[a] Proteins in cell-free culture fluids from wild-type strain Yb2 and mutant strain Yb2ΔgldK were analyzed with LC–MS/MS.
[b] T9SS CTD identified with BLASTP analysis. + indicates the protein has a CTD.
[c] Difference in protein secretion between Yb2ΔgldK and Yb2.

Table 2
Real-time qPCR verification of differentially expressed genes in mutant strain Yb2ΔgldK[a].

Gene ID	Product	Fold change
AS87_RS00815	Hypothetical protein	1.38
AS87_RS07305	Cytochrome c nitrate reductase small subunit	1.21
AS87_RS00555	RNA polymerase sigma 24 factor	1.08
AS87_RS02905	TonB-dependent sigma factor	0.73
AS87_RS08655	Tat pathway signal sequence domain-	0.72
AS87_RS08420	containing protein	0.69
AS87_RS00800	Hypothetical protein	0.68
AS87_RS02915	ABC transporter related protein	0.64
AS87_RS07980	Hypothetical protein	0.51
AS87_RS01190	Hep_Hag family protein	0.41
AS87_RS01370	Hypothetical protein	0.41
AS87_RS01365	Membrane protein	0.36
AS87_RS09460	Hypothetical protein	0.33
AS87_RS01360	Hypothetical protein	0.32
AS87_RS01355	Hemolysin D	0.31
AS87_RS02680	Transporter	0.29
AS87_RS01350	Hypothetical protein	0.27
AS87_RS07855	Heavy metal efflux pump, CzcA family	0.27
AS87_RS08825	ArsR family transcriptional regulator	0.24
AS87_RS09445	1- aminocyclopropane-1-carboxylate	0.23
AS87_RS05240	deaminase	0.18
	Hypothetical protein	
	Hypothetical protein	
AS87_RS08465	Gliding motility protein GldM	0.16

[a] Based on *R. anatipestifer*Yb2 genome (accession number: CP007204).

Academy of Agricultural Sciences (CAAS-XTCX2016011-04-8).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.03.006.

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