Attenuation of \textit{Yersinia pestis} \textit{fyuA} Mutants Caused by Iron Uptake Inhibition and Decreased Survivability in Macrophages

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\textit{Yersinia pestis} is the etiological agent of plague, a deadly infectious disease that has caused millions of deaths throughout history. Obtaining iron from the host is very important for bacterial pathogenicity. \textit{Y. pestis} possesses many iron uptake systems. Yersiniabactin (Ybt) plays a major role in iron uptake \textit{in vivo} and \textit{in vitro}, and in virulence toward mice as well. FyuA, a β-barrel TonB-dependent outer membrane protein, serves as the receptor for Ybt. In this study, we examined the role of the \textit{fyuA} gene in \textit{Y. pestis} virulence using different challenging ways and explored the underlying mechanisms. The BALB/c mouse infection assay showed that the virulence of the mutant strains (\textit{DfyuA} and \textit{DfyuAGCADel}) was lower when compared with that of the wild-type (WT) strain 201. Furthermore, the attenuation of virulence of the mutant strains \textit{via} subcutaneous and intraperitoneal challenges was far greater than that \textit{via} intravenous injection. Iron supplementation restored lethality during subcutaneous challenge with the two mutants. Thus, we speculated that the attenuated virulence of the mutant strains toward the mice may be caused by dysfunctional iron uptake. Moreover, \textit{DfyuA} and \textit{DfyuAGCADel} strains exhibited lower survival rates in murine RAW264.7 macrophages, which might be another reason for the attenuation. We further explored the transcriptomic differences between the WT and mutant strains at different temperatures and found that the expressions of genes related to Ybt synthesis and its regulation were significantly downregulated in the mutant strains. This finding indicates that \textit{fyuA} might exert a regulatory effect on Ybt. Additionally, the expressions of the components of the type III secretion system were unexpectedly upregulated in the mutants, which is inconsistent with the conventional view that the upregulation of the virulence genes enhances the virulence of the pathogens.

\textbf{Keywords: \textit{Yersinia pestis}, iron uptake system, Ybt system, \textit{fyuA}, virulence, pathogenicity}
INTRODUCTION

Iron is indispensable to almost all living things, but its availability is very low (Noinaj et al., 2010). In mammals, iron mainly exists in the form of Fe$^{3+}$-protein complexes (ferritin, transferrin, and lactoferrin), and the concentration of free iron ions does not exceed 10^{-18} M, which is too low to sustain bacterial growth and reproduction (Bullen et al., 1978). Different iron acquisition systems have been identified in various bacterial pathogens (Schaible and Kaufmann, 2004; Braun, 2005; Fetherston et al., 2010; Butt and Thomas, 2017; Dichtl et al., 2019).

*Yersinia pestis*, the causative agent of plague, possesses multiple iron transport systems, including two heme transporters named Hmu and Has, many ATP-binding cassette (ABC) transporters (Yfu, Yfe, and Yiu), several ferrous iron transporters (Yfe, Feo, Fet, etc.), and the yersiniabactin (Ybt) system, which play a major role in the virulence toward mice (Fetherston et al., 2010; Perry et al., 2015). Ybt has been identified in pathogenic *Yersinia* species (*Y. pestis, Y. pseudotuberculosis*, and *Y. enterocolitica*), and it serves as a siderophore, a class of low-molecular-weight (<1000 Da) non-protein compounds with high specificity and high affinity for Fe$^{3+}$ (Heesemann, 1987; Heesemann et al., 1993; Perry et al., 1999). Ybt consists of one salicylate, one thiazoline, and two thiazolidine rings, which have six coordinate binding sites for Fe$^{3+}$ (the formation constant for Fe$^{3+}$ is 4 × 10^{36})(Chambers et al., 1996; Gehring et al., 1998; Perry et al., 1999; Miller et al., 2006; Drechsel et al., 1995).

FyuA is a TonB-dependent β-barrel outer membrane receptor that acts as the dual receptor for Ybt and pesticin (Heesemann et al., 1993; Rakin et al., 1994; Lukacik et al., 2012). Ybt can capture Fe$^{3+}$ from lactoferrin and transferrin. Subsequently, the Ybt-Fe$^{3+}$ complex is transported into the cells via the outer membrane receptor FyuA. Upon reaching the periplasm, the complex is transported into the cytoplasm by two inner membrane YbtP/YbtQ (Perry and Fetherston, 2011). The TonB system is composed of ExbB-ExbD anchored to the inner membrane and the peripheral lipoprotein TonB. The system provides energy for TonB-dependent outer membrane receptors to transport nutrients, such as iron complexes and vitamin B12 (Schauer et al., 2008). In *Y. pestis*, Ton-box is located at the N-terminus of the FyuA plug domain, which is important for interactions with TonB-ExbB-ExbD (Rakin et al., 1994; Fetherston et al., 1995; Lukacik et al., 2012). *Y. pestis* possessing mutations in either fyuA or tonB displays similar defects in iron-deficient growth (Perry et al., 2003). Given its importance in transporting ferric iron, FyuA also plays a key role in the virulence of some pathogenic *E. coli* strains and *Yersinia*.

In this study, we constructed ΔfyuA (lacking fyuA) and ΔfyuA_gcadel (GCA three-base deletion at positions 915–917 of fyuA) mutants and compared them with the wild-type (WT) strain. Our results revealed that both mutants were less virulent toward HeLa cells and animals, which might be attributed to iron uptake deficiencies in the mutants.

MATERIALS AND METHODS

**Bacterial Strains and Culture Conditions**

The bacterial strains and plasmids used in this study are listed in **Supplementary Table 1**. Strain 201 belongs to the biovar Microtus and was isolated from *Microtus brandti* in Inner Mongolia, China. This strain is extremely lethal to mice but avirulent to humans (Fan Z, 1995). The strains were grown in Luria-Bertani (LB) broth (pH 7.4) or on LB agar plates at 26°C (*Y. pestis*) or 37°C (*E. coli*). The glycerol-preserved strains were inoculated into 5 ml of LB medium and allowed to be fully activated. The recovered strains were subjected to two consecutive passages, and the second-passage cultures were used in follow-up experiments. Chloramphenicol (Cm) was added to the media at 34 μg/ml when needed.

**DNA Preparation**

Plasmids and genomic DNA were extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) and QIAamp DNA Mini Kit (Qiagen, Germany) separately in accordance with the manufacturer’s instructions. Polymerase chain reaction (PCR) was performed with an automated thermal cycler (Bio-Rad T100™) using 1×Taq Master Mix (purple) (Biomed, China). The initial denaturation step (94°C, 5 min) was followed by 30 cycles of denaturation (95°C, 30 s), annealing (the temperature was set according to the primers used, 30 s), and extension (72°C, the time was set according to the length of the target gene), with a final extension step (72°C, 5 min).

**Construction and Identification of the Mutant Strains and Complementation Strains**

The upstream and downstream homology arms of fyuA were amplified from strain 201 using the primer pairs Pre-fyuA-F/R and Post-fyuA-F/R separately, and the amplification products were purified with QIAquick PCR Purification Kit (Qiagen, Germany). The pDS132 plasmids were digested with *Sph I*-HF (New England BioLabs, USA). The homology arms and digested pDS132 plasmids were ligated using 2×Seamless Cloning Mix (Biomed, China) at 50°C for 15 min, followed by introduction into S17λpir using a Bio-Rad Gene Pulser Xcell Electroporation System (2.5 kV, 25 μF, 200 Ω), thereby generating S17-pDS132-fyuA and S17-pDS132-fyuA_gcadel.

S17-pDS132-fyuA and strain 201 were grown in LB medium at 37°C and 26°C, respectively, to an OD_{600 nm} of 0.6–0.8. S17-pDS132-fyuA (1.5 ml) and 201 (100 μl) cultures were centrifuged at 4500 rpm for 5 min, resuspended in 50 μl of LB medium, and sufficiently mixed, following which the cells were pipetted onto a filter paper (45 μm) attached to the LB plate. The plate was incubated overnight at 26°C. The cells were then eluted with LB, coated on a Yersinia Selective Agar Base (Difco™, USA) plate containing 6.8 μg/ml chloromycetin, and incubated at 26°C for 4–5 days. Subsequently, 5–6 single colonies were selected and placed in LB medium and subjected to a shaking culture overnight at 26°C, coated on LB plates containing 7% sucrose for screening, and then incubated at 26°C for 3 days. PCR using the fyuA-seqF/R primers was performed for verification; the amplification products were then confirmed using DNA sequencing.

To construct the complemented strains, a PCR-amplified DNA fragment containing the fyuA coding sequences together with 300 bp and 283 bp of its respective upstream and downstream sequences was cloned into pACYC184 that had...
been digested with Hind III-HF and BamH I-HF (New England BioLabs, USA). The purified PCR products and digested pACYC184 were ligated using 2×Seamless Cloning Mix (Biomed, China) at 50°C for 15 min and then transferred into DH5α cells (Biomed, China). After DNA sequence verification, the recombinant plasmids expressing fyuA were introduced into ΔfyuA and ΔfyuAGCADfl separately, thus generating the complemented mutant strains ΔfyuA-Comp and ΔfyuAGCADfl-Comp. The primer sequences used are shown in Supplementary Table 2.

**Growth Curve Determination of Y. pestis In Vitro**

The Y. pestis strains were grown in LB medium at 37°C to an approximate OD620 nm of 1.0 (ca. 2 × 10⁸ CFU/ml). The bacterial cultures were diluted 1:20 in a 50 ml Erlenmeyer flask containing small glass beads and 20 ml of fresh LB and then incubated at 37°C with shaking at 220 rpm. The bacterial growth was monitored by measuring the absorbance at OD620 nm at 2 h intervals. The experiment included three independent biological replicates, and the results were expressed as mean ± standard deviation from three independent experiments. The growth curve determination of Y. pestis in TMH (0.1 mM FeSO₄ and 10 μM FeCl₃) medium (Zahorchak and Brubaker, 1982; Straley, 1986; Dreschsel et al., 1995) was performed at 26°C using the same method as above.

**Real-Time Cell Analysis (RTCA) Assay**

The HeLa cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ incubator. For this assay, 150 μl of DMEM (Gibco, Thermo Fisher Scientific, USA) containing 10% FBS was pipetted into each well of the E-plate connected to the RTCA iCELLigence system (ACEA Biosciences, San Diego, CA, USA), which was placed at 37°C in a 5% CO₂ incubator to measure the baseline. The concentration of the HeLa cells was adjusted to 5 × 10⁵ cells/ml, and 300 μl of it was pipetted into each well of the E-plate. The E-plate containing the cells was incubated for 30 min at 37°C in a 5% CO₂ incubator, followed by transfer to the RTCA iCELLigence system, which was maintained under the same condition to obtain a stable baseline (10 h). The harvested Y. pestis cells were resuspended in sterile phosphate-buffered saline (PBS) and used to infect the HeLa cells at a multiplicity of infection (MOI) of 10. The cells were incubated, and the cell index (CI) was measured at 2 min intervals.

**Protein Structure and Function Prediction**

Iterative Threading ASSEMBLY Refinement (I-TASSER, as “Zhang-Server”) (Yang and Zhang, 2015) is a hierarchical approach for protein structure prediction and structure-based functional annotation. Identification of the structural templates from the Protein Data Bank (PDB) (https://www.rcsb.org/) using the multiple threading approach LOMETS, with full-length omics models constructed using iterative template-based fragment assembly simulations is the first step. Insights into the function of the target are then derived by rethreading the 3D models via a protein function database, BioLiP (Yang et al., 2013). I-TASSER has been ranked as the No. 1 server for protein structure prediction in recent community-wide CASP 7–14 experiments.

**Mouse Infection**

Y. pestis was cultivated according to the above method to ca. 2 × 10⁹ CFU/ml. The cultures were centrifuged at 4500 rpm for 5 min, washed twice, and diluted in sterile PBS to the desired cell density. The concentration of the bacterial cells was determined via dropping on Hottinger’s agar plates. The female BABL/c mice (6–8 weeks old) were provided by Beijing Vital River Laboratory Animal Technology Co. Ltd. [laboratory animal permit no. SCXK (Jing) 2016-0006]. The animals infected with the WT and mutant strains received the injection of 100 μl cultures diluted with PBS to the desired concentration via three routes (subcutaneously, intravenously, or intraperitoneally). The mice that needed iron supplementation were injected (subcutaneously or intraperitoneally) with 50 μg of FeCl₃ diluted in sterile deionized and distilled water. Mortality was recorded continuously for 14 d. GraphPad Prism 5.0 software was used to draw the survival curves. The data were analyzed using log-rank (Mantel–Cox) test, with P < 0.05 considered statistically significant. In this study, all mice were handled as per the Guidelines for the Welfare and Ethics of Laboratory Animals of China.

**Ability of Y. pestis to Survive in the Blood and Serum of Mice**

Mouse blood and serum were provided by Beijing BioRab Technology Co. Ltd. A total of 950 μl of blood was pipetted into each well of a 24-well plate. The second-passage Y. pestis cultures were grown to ca. 2 × 10⁹ CFU/ml, centrifuged at 4500 rpm for 5 min, and resuspended in PBS. Subsequently, 50 μl of the bacterial suspension diluted 625 times (ca. 3.2 × 10⁵ CFU/ml) was pipetted into a 24-well plate to which 950 μl of blood was added in advance. The 24-well plate was placed at 37°C in a 5% CO₂ incubator. At 0, 2, 4, 6, and 8 h, the blood was mixed fully by pipetting, and 50 μl of it was pipetted into a 96-well plate to which 200 μl of PBS was added to each well in advance. Thus, a five-fold gradient continuous dilution was created until the number of bacterial colonies could be counted. The number of bacteria in blood was counted by dropping 40 μl of diluted blood onto Hottinger’s agar plates in triplicate. This experiment had three independent biological replicates, and the result was expressed as mean ± standard deviation (n = 3).

**Ability of Y. pestis to Survive in RAW264.7 Cells**

RAW264.7 cells were maintained in DMEM (Gibco, Thermo Fisher Scientific, USA) containing 10% FBS at 37°C in a 5% CO₂ incubator. Later, 500 μl of the cells were pipetted into 24-well plates at a concentration of 4 × 10⁵ cells/ml on the day before the infection. The second-passage Y. pestis cultures were grown to a bacterial concentration of approximately 2 × 10⁸ CFU/ml and were collected separately by centrifugation and resuspended in sterile PBS. The RAW264.7 cells were then infected with strain 201 at an MOI of 5, and 50 μg/ml of gentamycin was added to the
medium to kill the extracellular bacteria after 30 min of infection. At 0.5, 1, 2, 4 and 8 h post infection (hpi), the culture medium from each well was discarded and the cells were thoroughly washed three times in PBS. The infected RAW264.7 cells were then lysed by the addition of sterile 0.1% Triton X-100 for 15 min at room temperature to release the intracellular bacteria. The living engulfed bacteria were counted by dropping the diluted cell lysate onto Hottinger’s agar plates in triplicate. This experiment comprised three independent biological replicates, and the results were expressed as mean ± standard deviation (n = 3).

RNA Isolation, Sequencing, and Data Analysis

Y. pestis WT strain 201 and the ΔfyuA<sub>GCAdel</sub> mutant were grown at 26°C or 37°C in TMH to a bacterial concentration of approximately 2 × 10<sup>8</sup> CFU/ml, and each sample included three biological replicates. Total RNA was extracted using the PureLink<sup>™</sup> RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, USA) and then used for creating a cDNA library and deep sequencing. According to the values of fragments per kilobase of transcript per million mapped reads, the ratio of transcript levels between WT and ΔfyuA<sub>GCAdel</sub> groups was used as the logarithm to the base 2 (twofold change). For example, a twofold change value of 1.0 indicates twofold greater expression of a certain gene. The differential values of at least twofold were applied to analyze the differential expression of genes according to the Y. pestis 91001 genome annotation.

Quantitative Reverse Transcription PCR (qRT–PCR) Analysis

qRT–PCR was used to confirm the results of RNA-seq. RNA samples that were subjected to sequencing were reverse-transcribed to cDNA as templates using SuperSript<sup>™</sup> Reverse Transcription Kit (Invitrogen, Thermo Fisher Scientific, USA). The qRT–PCR analysis was performed based on SYBR Green I (Roche) fluorescence using Roche Light Cycler 480. Correlations between the sequencing data and the qRT–PCR results were calculated using linear regression. All primer pairs were designed to produce amplicons with expected sizes of 50–200 bp using the Primer Premier 5.0 software. The primer sequences used are shown in Supplementary Table 3.

Ethics Statement

The animal study was conducted in accordance with institutional guidelines and ethical regulations of the Beijing Institute of Microbiology and Epidemiology.

Statistical Analysis

All data of the three experimental groups were obtained from three independent experiments and expressed as mean ± standard deviation. If the data conformed to normality and homogeneity of variance, the one-way analysis of variance was performed, and the Student–Newman–Keuls–q test was applied for multiple comparisons. If the data did not conform to normality, the Kruskal–Wallis test was performed, and the comparisons were analyzed using the Nemenyi test. If the data conformed to normality but not to homogeneity of variance, the Brown–Forsythe test was performed. The log-rank (Mantel–Cox) test was performed for survival curve analysis. Statistical significance was set as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

RESULTS

Identification of the ΔfyuA and ΔfyuA<sub>GCAdel</sub> Mutants and Determination of Their Growth Curves

To explore the role of fyuA in the virulence of Y. pestis biovar Microtus strain 201, the suicide vector pDS132 was used to construct the mutants ΔfyuA and ΔfyuA<sub>GCAdel</sub>. The corresponding complemented strains were constructed (Supplementary Table 1) as described in Materials and Methods. The mutants were identified using PCR and sequencing (Supplementary Figure 1). In vitro growth curves constructed for strain 201 and ΔfyuA in LB medium and TMH (a defined medium) containing different concentrations and types of iron ions showed no significant differences (Figures 1A–D). This finding indicated that the loss of fyuA had limited effect on the growth of the mutant strains under conditions of abundant iron and nutrition. Thus, phenotypic analysis of the mutants in comparison with the WT strain should not have been influenced by the differences in growth rates. Interestingly, the mutants showed growth rates similar to those of strain 201 in iron-depleted medium (Figures 1E, F), which differed from the result of KIM6+ fyuA mutant that exhibited retarded growth in iron-deficient medium (Fetherston et al., 2010).

Attenuation of the Cytotoxicity of the Mutants toward HeLa Cells

RTCA can monitor infected HeLa cells inoculated onto a gold matrix by measuring the cell index (CI), an indicator that reflects cellular changes, including cell adhesion and cell number (Roshan Moniri et al., 2015; Cetin and Topcul, 2019). The CI readouts of the WT strain infected HeLa cells were significantly lower than those of the mutant strains infected cells infected with the mutant strains from 2h to 9h post infection (12h to 21h in the RTCA assay) (Figure 2). This result shows that the cytotoxicity of ΔfyuA and ΔfyuA<sub>GCAdel</sub> toward HeLa cells was attenuated, thus indicating that the fyuA gene is involved in the cytotoxicity of Y. pestis toward HeLa cells.

Prediction of Ligand Binding Sites of FyuA and FyuA<sub>Gln</sub>

The finding that the deletion of only three bases in fyuA at positions 915–917 can lead to attenuation of Y. pestis virulence is interesting. In this study, we predicted the structure and ligand binding sites of FyuA and FyuA<sub>Gln</sub> (glutamine at position 305 of the FyuA protein was absent) using I-TASSER and focused on differences in ligand binding sites between them. Functional templates of the target protein were comprehensively scored using COFACTOR and COACH based on the I-TASSER structure prediction. The predicted functional templates with the top five C-scores (C-score range of 0–1, where a higher score
indicates a more reliable prediction) of the ligand binding sites of FyuA and FyuAGlndel are depicted in Table 1. In the top five, three unique functional templates named 2grxA, 2W76B, and 2GSKA (PDB ID) were predicted for FyuA, which were missing in FyuAGlndel. The fifth functional template 2GSKA binds the ligand 2GSKA00. We searched PDB and found that the functional template is the BtuB : TonB complex. TonB, which possesses a single putative transmembrane helix, a proline-rich linker region, and a periplasmic C-terminal domain, couples the inner membrane proton-motive force (pmf) to the outer membrane transporter (Wiener, 2005). TonB-dependent outer membrane transporters possess a conserved motif named Ton-box (Lundrigan and Kadner, 1986; Schramm et al., 1987), which interacts with TonB during the active transport cycle, and deletion (or certain mutations) of the Ton-box stops the transport (Bell et al., 1990). Thus, we speculated that the deletion of glutamine (Gln) on FyuA damaged the TonB ligand binding sites that are necessary for iron transport, thus leading to attenuated virulence of ΔfyuAGCAdel mutant strains.

**Mutants Showed Different Virulence Toward Mice in Different Plague Models and Iron Accelerated Lethality During Their Subcutaneous Infection**

*Y. pestis* biовар Microtus 201 strains are highly virulent toward mice but avirulent to guinea pigs and other large animals. The *fyuA* gene plays an important role in the virulence of *Yersinia* spp. (Rakin et al., 1994; Brem et al., 2001). Based on the finding of lower cytotoxicity of ΔfyuAGCAdel toward HeLa cells, we challenged the mice via three routes. In groups of BALB/c mice (*n* = 10) infected with WT, ΔfyuA, or ΔfyuAGCAdel via intraperitoneal (intra) injection, ΔfyuA and ΔfyuAGCAdel displayed significantly attenuated virulence [P < 0.0001, Log-rank (Mantel–Cox) Test]. Additionally, the 14-day survival rates of mice challenged with ΔfyuA or ΔfyuAGCAdel mutants were 90% and 80%, respectively (Figure 3A). In groups of BALB/c mice (*n* = 10) infected with the WT or mutant strains via the intravenous route (septicemic plague model), the 14-day survival rate of mice injected with each mutant was 10% (Figure 3B).

This finding differs from that of another study in which an *fyuA* mutant was fully virulent in mice when administered intravenously (Bobrov et al., 2014; Perry et al., 2015). In a previous research, *Y. pestis* strains with mutations in *fyuA* showed losses of virulence of >1.13 × 10 6-fold and 33-fold upon subcutaneous and intranasal infections, respectively (Fetherston et al., 2010). In our research, a similar result was obtained when the mice were challenged subcutaneously (Figure 3C). Moreover, the complemented strains of the mutants showed virulence reversion on mice challenged through subcutaneous injection (Figure 3C).

The attenuation of nonpigmented KIM D27 strains, when administered subcutaneously, has long been attributed to deficient iron acquisition and metabolism, partially because intraperitoneal injection of inorganic iron appeared to restore virulence (Burrows and Jackson, 1956; Brubaker et al., 1965). This situation is also applicable to pneumonic plague models (Lee-Lewis and Anderson, 2010). The structure prediction using I-TASSER indicated that the attenuation of ΔfyuAGCAdel may be due to the blockage of iron transportation. To confirm this result, FeCl₂ was injected into mice throughout the experiment. The iron-treated mice were challenged with ΔfyuA and ΔfyuAGCAdel subcutaneously (bubonic plague model). The mice injected with...
50 µg of FeCl₂ subcutaneously and intraperitoneally twice daily exhibited the same clinical signs as those infected with the WT strains. All mice rapidly and consistently developed lethal infection and died within 6 days (Figure 3D). Meanwhile, the mice injected with 50 µg of FeCl₂ only intraperitoneally died within 14 days (Figure 3D), which is later than the mice infected with the WT strains. These results assert that fyuA is involved in the virulence of strain 201 and that the inhibition of iron uptake in the mutants might attenuate their virulence.

The formation constant of Ybt with ferric iron is $4 \times 10^{36}$; hence, Ybt can remove iron from transferrin and lactoferrin. Blood is abundant in iron, which is a component of hemoglobin. We speculated that the Ybt system plays a more important role than other iron uptake systems when the mice are challenged subcutaneously, in which Y. pestis went through an iron-deficient environment. However, other systems may be more effective in acquiring iron once bacteria have disseminated via the bloodstream. To prove this hypothesis, we compared the abilities of Y. pestis 201 and mutant strains to survive in mouse blood or serum, and their survival rates were calculated by counting the colonies after incubation in blood or serum for 0, 2, 4, 6, and 8 h. The results showed that the total bacterial counts of 201 and mutant strains in blood (Figure 4A) or serum (Figure 4B) did not differ significantly, which denoted that the deletion or mutation of fyuA does not affect the reproduction of Y. pestis in blood or serum. This finding supports our hypothesis that the Ybt system may not work in the septicemic plague model. This result is consistent with that obtained for mice challenged by intravenous injection. Because the growth and reproduction of the fyuA mutant strains are not affected in the blood, the virulence of the mutant strain toward mice when administered intravenously is only slightly weakened.
Survival Rate of ΔfyuA and ΔfyuAGCAdel Mutants Was Lower Than That of Strain 201 in RAW264.7 Cells

The ability of Y. pestis to survive in macrophages plays an important role in the early stages of host infection (Straley et al., 1984). In this study, we compared the ability of 201 and mutant strains to survive in RAW264.7 cells to explore whether the attenuation of the mutant strains is related to their weakened ability to survive in the macrophages. RAW264.7 cells were infected continuously for 8 h with ΔfyuA, ΔfyuAGCAdel, and WT strains at an MOI of 5. Subsequently, their survival rates were measured using a gentamycin protection assay, followed by plating the cell lysates on Hottinger’s plates. The number of live bacteria in RAW264.7 cells at 0.5 hpi was designated as the initial value, and the survival rates at 1, 2, 4 and 8 hpi were calculated by dividing the corresponding live bacterial cell number with that at 0.5 hpi. The percentage survival of 201, ΔfyuA, and ΔfyuAGCAdel decreased to approximately 70%, 45%, and 55%, respectively, in RAW264.7 cells at 1 hpi. This percentage continued to decrease to approximately 50% and 35%–40% at 2 hpi. This trend of WT exhibiting higher survival rates than the mutant strains in RAW264.7 cells continued until 6 hpi; neither WT nor mutant strains were detected in the cells at 8 hpi (Figures 4C, D). These results indicate that fyuA plays an important role in the early stage of Y. pestis infection, and the weakened ability of the mutant strain to survive in macrophages may contribute to its attenuated virulence.

### Table 1

| FyuA | FyuAGCAdel |
|------|------------|
| C-score | PDB | Lig | Hit | Name* | C-score | PDB | Lig | Hit | Name* |
| 0.05 | 1ujwA | LIM | 147, 148, 599, 611, 642, 644 | 0.06 | 1ujwA | LIM | 147, 148, 598, 610, 641, 643 |
| 0.05 | 2grxA | FTT | 214, 263, 278, 280, 301, 330 | 0.05 | 3m8aA | C8E | 321, 361, 363, 373, 374, 375, 376 |
| 0.05 | 1nqA | C8E | 380, 378, 379, 380, 407, 409, 410 | 0.04 | 1NQHA | 1NQHA00 | 60, 61, 62, 63, 64, 65, 67, 76, 77, 78, 79, 80, 229, 230, 231, 232, 287, 297, 544, 545, 623, 624, 625, 632 |
| 0.03 | 2W76B | 2W76B01 | 562, 563, 564, 565, 567, 599, 609, 610, 611, 644, 645, 646 |
| 0.03 | 2GSKA | 2GSKA00 | 60, 61, 62, 63, 64, 65, 67, 76, 77, 78, 79, 80, 229, 230, 231, 232, 287, 296, 298, 54, 546, 624, 625, 626, 633 |
| 0.03 | 1nqA | C8E | 359, 377, 378, 406, 408, 409, 410 |
| 0.03 | 2FCPA | 2FCPA02 | 218, 262, 263, 280, 302, 304, 329 |

*Lig Name is name of possible binding ligand.
Comparative Transcriptomics of 201 and ΔfyuA_GCA_del Cultured at 26°C or 37°C

To better explore the influence of GCA deletion or fyuA gene knockout with GCA deletion on *Y. pestis*, we compared the transcriptomes of strain 201, ΔfyuA, and ΔfyuA_GCA_del cultured at 26°C or 37°C (Figure 5). The RNA-seq data were confirmed using the qRT-PCR analysis of several genes (Supplementary Table 4), and the samples used in RNA-seq library construction were used as templates in qRT-PCR. The correlation coefficients (R²) between the RNA-seq and qRT-PCR results were >0.90 for the RNA samples obtained at 26°C and 37°C (Figure 5C and Supplementary Table 4). These results signify the high reliability of the RNA-seq data described in this paper.

As shown in Figure 5A, 766 genes of ΔfyuA and 887 genes of ΔfyuA_GCA_del cultured at 26°C were differentially expressed when compared with strain 201, and 494 of these genes were shared in these two mutant strains. While when cultured at 37°C, the gene numbers were 1438, 1223 and 902 respectively. The clustering heatmap shown in Figure 5B also revealed similar expression patterns of ΔfyuA and ΔfyuA_GCA_del mutant strains when cultured at these two temperatures.

The results of RNA-seq showed that genes in ΔfyuA_GCA_del related to the Ybt system, such as ybtX, ybtS, YbtQ, YbtU, YbtT, YbtE, irp2, irp1, and fyuA, were downregulated compared with those of strain 201 at both 26°C and 37°C (Table 2). Thus, fyuA may have some potential regulatory effects on the Ybt system. Moreover, the genes of the type III secretion system (T3SS) in ΔfyuA_GCA_del, such as lcrV, yscA, yscX, yopT, yopE, and yopB, were upregulated compared with those in strain 201 at 37°C (Table 3).

**DISCUSSION**

To investigate the role of fyuA in the virulence and pathogenicity of *Y. pestis* biovar Microtus strain 201, we constructed ΔfyuA and
DfyuA mutants. We compared the phenotypes of the two mutants and strain 201 in terms of their virulence toward mice and viability in mouse blood, serum and in RAW264.7 cells. In previous studies, the fyuA gene has been shown to be associated with the growth and virulence of Y. pestis (Heesemann et al., 1993; Rakin et al., 1994; Fetherston et al., 2010). In this paper, we have confirmed that a GCA deletion of fyuA, which cause a Glu736 deletion in FyuA, could also lead to similar results as ΔfyuA. KIM6+ strains with fyuA mutation showed a significant growth defect compared with the WT strain in deferrated PMH2 with 10 μM FeCl₃. The mutants displayed virulence losses of >1.13 × 10⁶-fold and 33-fold when the mice were injected subcutaneously and intravenously, respectively (Fetherston et al., 2010). Our findings alluded that there were no significant differences in growth between strain 201 and the two mutants in LB or TMH medium containing different types and concentrations of iron. Thus, the phenotypic difference among the strains observed in this study was not influenced by their growth.

We compared the cytotoxicity of the WT and mutant strains toward HeLa cells. The results showed that the cytotoxicity of ΔfyuA and ΔfyuAGCAdel mutant strains toward HeLa cells was reduced. In mouse infection experiments, we also found that the virulence of fyuA mutants was attenuated, with the degree of attenuation differing among the different challenge routes. The

FIGURE 5 | Comparative transcriptomics of strain 201 and ΔfyuAGCAdel cultured at 26°C or 37°C. (A) Venn diagrams showing overlapping of the gene expression profiles between different comparison groups. (B) Clustering analysis of differential gene expression between WT and mutant strains. Different colors show different gene expression levels, with high expression being colored red and low expression being colored green. (C) Correlations among the expression levels of 17 genes measured with RNA-seq and qRT-PCR were analyzed using linear regression.

TABLE 2 | The genes of Ybt system were down-regulated in ΔfyuAGCAdel in comparison with 201-WT.

| gene_id     | log₂FoldChange 26°C | log₂FoldChange 37°C | gene_name  | gene_description                  |
|-------------|---------------------|---------------------|------------|-----------------------------------|
| YP_RS08660  | -2.9                | -2.5                | ybtS       | yersiniabactin biosynthesis salicylate synthase YbtS |
| YP_RS08655  | -3.1                | -2.4                | ybtX       | yersiniabactin-associated zinc MFS transporter YbtX |
| YP_RS08650  | -2.1                | -2.3                | ybtQ       | yersiniabactin ABC transporter ATP-binding/permease protein YbtQ |
| YP_RS08645  | -2                  | -3.9                | ybtP       | yersiniabactin ABC transporter ATP-binding/permease protein YbtP |
| YP_RS08639  | -4.2                | -4                  | ipj2       | yersiniabactin non-ribosomal peptide synthetase HMWP2 |
| YP_RS08630  | -3                  | -2.8                | ipj1       | yersiniabactin polyketide synthase HMWP1 |
| YP_RS08625  | -3.5                | -2.9                | ybtU       | yersiniabactin biosynthesis oxidoeductase YbtU |
| YP_RS08620  | -4.5                | -3.1                | ybtT       | yersiniabactin biosynthesis thioesterase YbtT |
| YP_RS08615  | -2.9                | -2.6                | ybtE       | yersiniabactin biosynthesis salkyl-AMP ligase YbtE |
| YP_RS08610  | -4.3                | -5                  | fyuA       | siderophore yersiniabactin receptor FyuA |
The genes of type III secretion system were up-regulated in ΔfyuA \(_{GCAdel}\) in comparison with 201-WT at 37°C.

| gene_id   | log\(_2\)FoldChange | gene_name   | gene_description                               |
|-----------|----------------------|-------------|-----------------------------------------------|
| YP_RS21370| 2.1                  | yerA        | YopE transcriptional regulator yerA           |
| YP_RS21385| 1.5                  | yapE        | type III secretion system effector GTPase activator YopE |
| YP_RS21390| 1.8                  | yapT        | T3SS effector cysteine protease YopT          |
| YP_RS21405| 1.4                  | sycT        | type III secretion system chaperone SycT      |
| YP_RS21420| 2.1                  | yapD        | type III secretion system leucine rich repeat protein |
| YP_RS21425| 1.4                  | yapB        | type III secretion system translocon subunit YapB |
| YP_RS21440| 1.5                  | lcrH        | type III secretion system chaperone LcrH      |
| YP_RS21455| 1.7                  | lcrV        | type III secretion system protein LcrV       |
| YP_RS21460| 1.8                  | lcrG        | type III secretion protein LcrG              |
| YP_RS21475| 1                    | lcrR        | type III secretion system regulator LcrR      |
| YP_RS21480| 1.9                  | yscA        | type III secretion system chaperone YscA      |
| YP_RS21490| 1.9                  | yscX        | type III secretion system protein YscX       |
| YP_RS21505| 1.5                  | sycN        | type III secretion chaperone YscN            |
| YP_RS21510| 1.2                  | yscY        | type III secretion system gatekeeper subunit YscY |
| YP_RS21515| 1.2                  | yopA        | type III secretion system gatekeeper subunit YopA |
| YP_RS21520| 1.1                  | yopN        | type III secretion system translocon subunit YapN |
| YP_RS21525| 1                    | yopB        | type III secretion system export apparatus protein Scrl |
| YP_RS21530| 1.1                  | yscY        | type III secretion system protein YscY       |
| YP_RS21540| 1.8                  | yapE        | type III secretion system chaperone YscE      |
| YP_RS21550| 1.3                  | yscB        | type III secretion system inner membrane ring subunit ScrlD |
| YP_RS21560| 1.2                  | -           | YscE family type III secretion system needle protein co-chaperone |
| YP_RS21570| 1.2                  | -           | type III secretion system needle filament subunit ScrlF |
| YP_RS21580| 1.2                  | -           | YscG family type III secretion protein       |
| YP_RS21590| 1.1                  | -           | type III secretion system sorting platform protein YscK |
| YP_RS21600| 1                    | yscK        | T3SS effector tyrosine-phosphatase YopH       |
| YP_RS21610| 1.1                  | yscJ        | type III secretion system effector acetyltransferase YopJ |
| YP_RS21620| 1.4                  | yscI        | T3SS effector protein kinase YopQ/YpkA        |

Mutant strains were highly attenuated when administered via the subcutaneous and intraperitoneal routes.

I-TASSER was used to predict the ligand binding site of FyuA and the mutant protein. The results implied that FyuA\(_{GCAdel}\) lacks the binding site named 2GSKA, which plays an important role in combining with TonB in the functional model (Shultis et al., 2006). The inner membrane protein TonB couples the inner membrane pmf to the outer membrane, and the protein consists of a single putative transmembrane helix, a proline-rich linker region, and a periplasmic C-terminal domain (Wiener, 2005). The C-terminal domain of TonB binds directly to a conserved region, named Ton-box, in the outer membrane transporter (Shultis et al., 2006). Ton-box interacts with TonB during the active transport cycle. Several studies have asserted that mutations in Ton-box prevent the transport of iron and bacteriocins (Cascales et al., 2007). The structure of FyuA in \(Y.\) \(pestis\) has been characterized and possesses features typical of the TonB-dependent transporter family, including a membrane-spanning 22-strand \(\beta\)-barrel whose pore is blocked by a plug domain (Lukacik et al., 2012). Thus, we speculated that the mutation of FyuA\(_{GCAdel}\) damaged the TonB ligand binding sites relevant to iron transport, thereby leading to the attenuated virulence of ΔfyuA\(_{GCAdel}\) mutant strains. Our hypothesis was confirmed by the increased lethality during subcutaneous infection with ΔfyuA\(_{GCAdel}\) mutant along with iron supplementation.

Iron uptake from the host is vital for the pathogenicity of \(Y.\) \(pestis\). The \(fyuA\) gene is located on the HPI island of the chromosomal \(pgm\) locus. In previous studies, both Ybt biosynthetic/transport mutants and \(\Delta pgm\) strains have been shown to be essentially avirulent in a bubonic plague model (Fetherston et al., 2010). Restoration of the virulence of the \(\Delta pgm\) mutant strains in bubonic and pneumonic plague models was achieved via the intraperitoneal injection of inorganic iron (Burrows and Jackson, 1956; Brubaker et al., 1965; Lee-Lewis and Anderson, 2010). In our study, the virulence of the \(fyuA\) mutant strains in the bubonic plague model was also restored by iron supplementation to the mice. This observation reminded us that the different degrees of attenuation of the mutant strains when administered via different routes may be related to the uneven distribution of iron in the mice. Furthermore, strain 201 and the mutants had similar viabilities in mouse blood, which may be the reason why the mutants exhibited only a mild attenuation intravenously. Une and Brubaker have also proven that the \(\Delta pgm\) mutant is fully virulent in mice using a septicemic plague model (Burrows and Jackson, 1956; Une and Brubaker, 1984). Consequently, we speculated that the Ybt system acquiring iron from transferrin and lactoferrin plays a more important role than the other systems in the bubonic plague model. However, other iron uptake systems may be more effective in acquiring iron once the bacteria have disseminated via the bloodstream. This point may be supported by the reported finding that a researcher suffering from hemochromatosis was infected with an attenuated nonpigmented \(Y.\) \(pestis\) (designated UC91309) and died of septicemic plague (Frank et al., 2011).

The results of RNA-seq showed that the expression levels of the related genes in the Ybt system of ΔfyuA\(_{GCAdel}\) were significantly downregulated. The decreased synthesis of Ybt also inhibited iron uptake, which may be responsible for ΔfyuA\(_{GCAdel}\) attenuation. The mechanisms behind the
involvement of FyuA in regulating the Ybt system need to be further explored. Additionally, the expression of genes encoding components of the T3SS in the mutant strains was upregulated in comparison with that of WT at 37°C, thus indicating that FyuA also has a potential role in regulating the T3SS.

Considering that the viability of Y. pestis in macrophages has a vital role in the early stages of host infection (Straley et al., 1984), we compared the survivability of 201 and mutant strains in RAW264.7. The results showed that the survival rates of ΔfyuA and ΔfyuAGCAdel mutants were lower than that of strain 201, which contributed to the attenuation of ΔfyuA and ΔfyuAGCAdel mutant strains.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE198937.

ETHICS STATEMENT

The animal study was reviewed and approved by Beijing Institute of Microbiology and Epidemiology.

AUTHOR CONTRIBUTIONS

YS, RY, and ZQ conceived and designed the study. YLC, KS, XC, RL, QZ, YH, and YT performed the experiments and data analysis. YLC, YJC, and YB performed bioinformatics analysis. YLC, KS, RY, and YS wrote the original draft. YLC, KS, RY, ZD, ZQ, and YS reviewed and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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