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Research Article

Identification and Pathogenicity of Emerging Fish Pathogen *Acinetobacter johnsonii* from a Disease Outbreak in Rainbow Trout (*Oncorhynchus mykiss*)

Baoliang Bi,[1] Yin Yuan,[1] Dan Jia,[1] Wansheng Jiang,[2] Hui Yan,[1] Gailing Yuan,[3] and Yu Gao [1]

[1]*Key Laboratory for Plateau Fishery Resources Conservation and Sustainable Utilization of Yunnan Province, College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China*
[2]*Hunan Engineering Laboratory for Chinese Giant Salamander's Resource Protection and Comprehensive Utilization, Jishou University, Zhangjiajie 427000, China*
[3]*College of Fisheries, Hubei Provincial Engineering Laboratory for Pond Aquaculture, Huazhong Agricultural University, Wuhan 430070, China*

Correspondence should be addressed to Yu Gao; gaoyu@ynau.edu.cn

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In November 2017, a group of farmed rainbow trout (*Oncorhynchus mykiss*) died in Yunnan Province, China, likely due to infection. These rainbow trout exhibited slow movement, no feeding, dark body color, exophthalmia, and occasional ulcers on the body surface. Pathogens were isolated from diseased rainbow trout livers, head kidneys, spleens, and eyes, and the strain was preliminarily identified as *Acinetobacter johnsonii* based on morphology, biochemical tests, and 16S rRNA and rpoB sequences. The isolated strains were highly sensitive to florfenicol, ciprofloxacin, oxolinic acid, and norfloxacin but resistant to ampicillin, doxycycline, sulfadiazine, thiamphenicol, and sulfamethazine. The selected isolate was performed for the experimental infection of rainbow trout to confirm its pathogenicity. Experimentally infected fish showed disease symptoms similar to those observed in fish naturally infected with these bacteria. Vacuolar degeneration was prevalent in the liver and spleen of diseased fish. Cytoplasm volume was very high in the lymphocytes of the head kidney. The protein structure and topology of monomeric outer membrane protein A (OmpA), outer membrane protein 34 (Omp34), and a nucleoside-specific outer membrane transporter protein Tsx (OmpTsx) of *A. johnsonii* were predicted by AlphaFold 2. The predicted local distance difference test (pLDDT) score was used for the assessment of structure prediction. Comprehensive analysis of antigenic determinants of the OMP family member, OmpA, contains three antigenic determinants, which are 90% similar with epitopes of *A. baumannii*. OmpA is a recombinant protein with 35 kDa expressed in the *Escherichia coli* system. Based on these findings, *A. johnsonii* is regarded as an emerging opportunistic pathogen in farmed rainbow trout. OmpA protein can be used as a subunit vaccine candidate molecule of *A. johnsonii*.

1. Introduction

Acinetobacter spp. are a group of aerobic, non-fermentative, Gram-negative bacteria that are widely distributed in diverse environments, including soil, water, sludge, and human skin [1, 2]. *Acinetobacter* is generally considered to be a conditional pathogen, often causing serious infections in humans [3]. They are very persistent in the environment, and they also have strong viability and multidrug resistance [4]. For example, with the extensive use of broad-spectrum antibiotics in the clinic in recent years, *A. baumannii* has shown an extraordinary ability to develop resistance to all antimicrobials, which is a serious threat to human health [5]. Additionally, *A. lwoffii*, *A. junii*, *A. pittii*, and *A. baumannii* are now considered to be fish pathogens [6–9].

A number of bacteria produce a similar syndrome, which is generically characterized by external reddening and

hemorrhage in the peritoneum, body wall, and viscera [10–12]. During intensive aquaculture rearing, environmental stress factors such as hypoxia, abnormal pH, and high population density generate an optimal setting for pathogens to thrive [13]. *Acinetobacter* spp. are widely distributed and have a wide range of hosts. They can infect marine and freshwater fishes of all ages [6, 8, 14], and they cause serious losses to the salmon and trout industries every year [15]. A sudden change in water temperature, especially in early to midsummer that corresponds to the conditions for bacterial disease occurrence, results in high fish mortality and great economic losses to farmers. Thus, it is imperative to study the causes of fish deaths and develop corresponding countermeasures.

Outer membrane proteins (OMPs), as important immunogenic proteins of Gram-negative bacteria, not only play an important role in the stability of cell structure but also are considered one of the virulence factors of *Acinetobacter* [16, 17]. OMPs can act as an adhesion molecule (haemagglutinin) or as a bacterial enzyme or toxin, which could be used as potential vaccine targets [18]. Several studies have shown that bacterial OMPs serve as a vaccine for immunization against bacteria infecting fish [19]. Additionally, targeting OMPs, these membrane-bound porins produce channels to export antibacterial agents outside the cell, thus resulting in drug resistance [20, 21].

Rainbow trout (*Oncorhynchus mykiss*) is a cold-water fish that is one of the most extensively farmed fish worldwide. However, the expansion of rainbow trout farming coupled with insufficient attention by farmers to comprehensive regulation of water quality has led to the deterioration of aquatic environmental conditions. As a result, bacterial diseases have become a key factor for restricting the healthy development of the rainbow trout breeding industry [22]. Until now, rainbow trout can be affected by a range of bacterial and viral pathogens and parasites, such as *Aeromonas salmonicida*, infective pancreatic necrosis virus, and *Ichthyophthirius multifilis* [23]. Some cases of *Acinetobacter* spp. infection in farmed fish have been reported in Poland and China [6, 8, 15, 24]. However, there is still limited information on the virulence factors of *Acinetobacter* and the role of *Acinetobacter* in fish pathology.

In this study, *Acinetobacter johnsonii* was isolated from infected rainbow trout from a farm in plateaus areas, identified by the morphological observation, biochemical identification, and *16S rRNA,* and *rpoB* gene sequence analysis. The complete open reading frames of the outer membrane proteins A (OmpA), outer membrane proteins 34 (Omp34, also known as Omp34 kDa and Omp33–36), and a nucleoside-specific outer membrane transporter protein Tsx (OmpTsx) were cloned, and OmpA recombinant plasmid was expressed in the *Escherichia coli* system. Additionally, the ten antibiotic agents against the bacterial strain were also assayed for minimal inhibitory concentrations (MIC). The aim of the study was to find a means for effective control of *Acinetobacter* spp. infections in rainbow trout and evaluation of risk associated with these microorganisms in fish farms.

2. Materials and Methods

2.1. Sampling. On November 10, 2017, approximately 20 diseased rainbow trout (20–23 cm length, 120–140 g) were collected at Xundian Farm, Kunming, Yunnan Province, China, and sent to our laboratory for diagnosis. Fish were maintained and handled according to the guidelines approved by the Institutional Animal Care and Use Committee at Yunnan Agricultural University (Kunming, China). The gill and body surface of each fish were examined microscopically for the presence of parasites and bacteria. The body surface then was swabbed using 70% ethyl alcohol to decontaminate the normal external bacterial flora. The liver, head, kidney, spleen, and eye were removed, and tissue was aseptically inoculated on brain heart infusion (BHI). After the incubation at 28 C for 24 h, bacteria isolated from fish were subcultured under the same conditions to check the purity of isolation.

2.2. Isolation and Identification of Bacteria. Pure culture colonies were grown in BHI at 28°C for 24 h and collected into 1.5 mL centrifuge tubes. The colonies were then characterized using bacterial biochemical reaction tubes and following the manufacturer's protocol (Hangzhou Microbial Reagent Co., Ltd., Hangzhou, China).

The DNA was extracted according to the manufacturer's protocol (Aidlab Biotechnologies Co., Ltd., Beijing, China). The 16S rRNA and rpoB sequences were amplified by PCR using universal primers (Table 1). PCR reactions were performed on a Bio-Rad T100™ (Bio-Rad Laboratories, Foster City, CA, USA) with of 50 μL of reaction mixture consisting of 25 μL of master mix (Tsingke Bioscience and Technology Company, Beijing, China), 1 μL of 10 μM forward and reverse primers, respectively, 2 μL of DNA template, and nuclease-free water to fill the remaining volume.

The 16s rRNA amplification reaction was initiated with a denaturation step at 94°C for 3 min followed by 30 cycles, each including denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 2 min. The PCR was completed with a 10 min extension at 72°C. The rpoB amplification reaction was initiated with a denaturation step at 96°C for 2 min followed by 30 cycles, each including denaturation at 96°C for 10 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min. The PCR was completed with a 10 min extension at 72°C.

The PCR products were electrophoresed on a 1% agarose gel and purified with an agarose gel purification kit (Aidlab Biotechnologies). The purified product was then ligated with the pClone007 vector (Tsingke), and ligation mixtures were used to transform competent *E. coli* DH5α. Selection for transformed colonies occurred on LB ampicillin (100 mg/mL)/X-Gal/IPTG plates. White colonies were purified and sequenced at Tsingke Bioscience and Technology Company. The sequences were deposited into the NCBI GenBank (accession number: OP271698- OP271707).

2.3. MIC Tests. The strains named Ajo2017L-9, Ajo2017L-10, Ajo2017E-1, and Ajo2017E-2 were randomly selected for MIC tests among 10 isolated strains. The following 10

Table 1: The primers were used in this study.

Gene symbols	Gene description	Primers	Sequences (5′-3′)
16S rRNA	16S ribosomal RNA	27F	AGAGTTTGATCMTGGCTCAG
		1492R	TACGGYTACCTTGTTACGACTT
rpoB	RNA polymerase beta subunit	Ac696F	TAYCGYAAAGAYTTGAAAGAAG
		Ac1093R	CMACACCYTTGTTMCCRTGA
OmpA	Outer membrane protein A	Forward	ATGCGTGCATTAGTTA
		Reverse	TTATTGTTTAGCATAAATG
Omp34	Outer membrane protein 34	Forward	ATGAAAAAACTTGGTTTAGC
		Reverse	TTAGAAACGGAATTTAGCAT
OmpTsx	Ion channel protein Tsx	Forward	TTAGAAGTGGTATTTAACCA
		Reverse	ATGCAATTAAAGCAACTTGC

antimicrobial agents were used to assay: ampicillin sodium salt (0.976–50 mg/L), neomycin sulfate (0.048–25 mg/L), ciprofloxacin (0.048–25 mg/L), doxycycline (0.488–250 mg/L), norfloxacin (0.048–25 mg/L), florfenicol (0.048–25 mg/L), sulfadiazine (0.048–25 mg/L), oxolinic acid (0.048–25 mg/L), thiamphenicol (0.976–50 mg/L), and sulfamethazine (0.976–50 mg/L). The antimicrobial agents were purchased from Solarbio (Solarbio, China).

The macrodilution method was used to determine the MIC value of the antibiotic agent tested. Briefly, a series of two-fold dilutions of the antibiotic agents were prepared in BHI. Pure culture colonies were grown in the BHI medium at 28°C for 24 h and then diluted at 2×10^8 colony-forming units (CFU) mL^{-1} using 0.65% NaCl. Next, 10 μL of bacterial suspension and 2 mL of antibiotic agent dilution were added to each glass tube. The completed titration was incubated at 100 rpm for 24 h at 28°C. The MIC was defined as the lowest concentration with no visible growth. The MIC breakpoint of antimicrobial agents refers to the Clinical and Laboratory Standards Institute (CLSI) [25]. For antimicrobial agents that CLSI does not have a reference, published information of the MIC breakpoint was adapted as their structural analogs [26–28].

2.4. Infectivity Experiment. Healthy rainbow trout (20–23 cm length, 120–140 g) were kept in two fiberglass tanks with cylindroconical bottoms (diameter: 1.2 m, water volume: 650 L). Water was recirculated through the filter at a rate of 4 L/min and maintained at an average temperature of 18°C. The fish were fed twice a day to satiation with a commercial feed for rainbow trout. These fish had no history of disease or abnormality and were acclimated for 14 days before challenge. The pathogen challenge experiments were performed according to the guidelines approved by the Institutional Animal Care and Use Committee at Yunnan Agricultural University (Kunming, China).

During the challenge experiment, 150 fish were randomly distributed into 15 tanks (10 fish per tank, 3 tanks per group) with a total volume of 600 L. The Ajo2017L-10 strain was diluted in 0.65% NaCl solution to achieve concentrations of 2.5×10^9, 2.5×10^8, 2.5×10^7, and 2.5×10^6 CFU mL^{-1}. In order to test Koch's postulate, 10 fish in each of 5 groups were injected at the base of pectoral fin with 0.2 mL bacterial suspension or 0.65% NaCl solution (mock challenge). The infectivity study lasted for 15 days.

The liver, kidney, and spleen from dying fish were aseptically inoculated on BHI. These tissues of fish from the control group and each treatment group were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, serially sectioned, and stained with hematoxylin eosin. After incubation at 28°C for 24 h, bacteria isolated from fish were subcultured under the same conditions to check purity of the isolation. Pure culture colonies were identified by 16S rRNA sequence.

2.5. Omp Family Member Clone and OmpA Prokaryotic Expression. The DNA was extracted according to the manufacturer's protocol (Aidlab Biotechnologies Co., Ltd., Beijing, China). The OmpA, Omp34, and OmpTsx sequences were amplified using PCR with specific primers, respectively (Table 1). PCR reactions and the clone process were similar to 16s rRNA and rpoB cloning. The sequences were deposited into the NCBI GenBank (accession number: OP312670- OP312672).

Using homologous recombination, pET32a-OmpA recombinant plasmids were constructed, and the recombinant plasmids were used to transform into competent *Escherichia coli* BL21. After pET32a-OmpA identification, the expression of recombinant protein was induced by IPTG during the period of logarithm growth of bacteria. The induction conditions were 1 mM IPTG at 25°C for 5 h. The bacteria were centrifuged at 5000 × g for 10 min at 4°C. The expression of recombinant protein was detected by SDS-PAGE.

For a large amount of expression, the recombinant strain was inoculated in 600 mL LB medium containing ampicillin (100 μg/mL) after the expanded culture of recombinant strain. 1 mM IPTG was added in the medium when OD600 reached 0.8, continue shaking culture at 37°C 200 rpm for 4 h. The bacterial medium was broken by ultrasonic after induction and then centrifuged at 4°C 5000 g for 10 min. The recombinant protein was purified by nickel affinity chromatography and detected by SDS-PAGE. We freeze-dry and store at −80°C.

2.6. Phylogenetic Analyses. The 16S rRNA and rpoB gene sequences were analyzed using the BLAST search function of the NCBI database for preliminary identification. The OmpA, Omp34, and OmpTsx amino acid sequences were

deduced using SeqEdit, Lasergene software (DNASTAR, Madison, WI, USA). Coding sequences were aligned based on their amino acid sequences with their respective orthologs. Neighbor-joining trees (NJ) with 1000 bootstrap replications were constructed using ClustalW as implemented in MEGA X [29] to visualize similarity and sequence divergence. For the concatenated datasets, only genes that were available for outgroup species were used.

2.7. Protein Structure Prediction. AlphaFold 2 (version 2.2.2) was used to predict the protein structure and monomer model. The model parameters were all default parameters. The prediction was conducted using the all-protein database. The database version: UniProt was 2022-08-03, PDB mmCIF was 2022-08-05, and the other databases were official default. We also collected the predicted local distance difference test (pLDDT) values for each structure. pLDDT values above 70 indicate generally good backbone prediction.

The protective antigens of OmpA, Omp34, and OmpTsx were predicted by VaxiJen [30]. The prediction of hydrophilicity, flexibility surface accessibility of protein was analyzed by the immune epitope database (IEDB). Antigenic peptides are determined using the method of Kolaskar and Tongaonkar [31], available at https://imed.med.ucm.es/Tools/antigenic.pl.

3. Results

3.1. Clinical Symptoms of Diseased Rainbow Trout. There was approximately 30% of total mortality from June to October in the farm. The death rainbow trout were mainly juvenile fish with 5–30 cm body length. The infected rainbow trout in the farm exhibited slow movement, no feeding, dark body color, exophthalmia, and occasional ulcers on the body surface. After dissection, infected fish of field investigation showed a pale liver, empty stomach, ascites, occasional intestinal edema, and hemorrhage resembling the septicaemic form of infection (Figure 1).

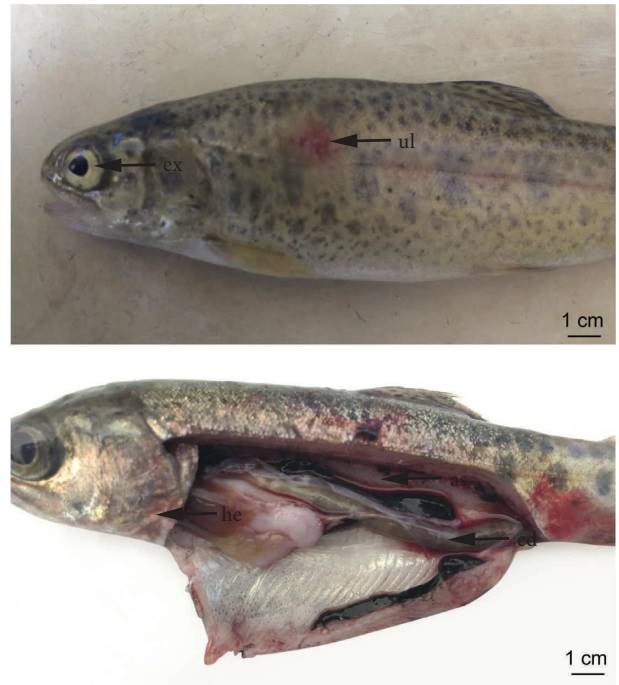

Figure 1: Clinical symptoms of diseased rainbow trout from a farm, Xundian County, Yunnan province, China: as: ascites; ed: intestinal edema; ex: exophthalmia; he: mild hemorrhage; ul: mild ulceration.

3.2. Physiological and Biochemical Properties of Isolated Strains. Pure colonies of Gram-negative bacteria were isolated on BHI from the livers (2 strains) and eyes (8 strains) from 20 diseased fish samples. After growth on the BHI medium for 24 h, the colonies were flat with smooth surfaces and neat edges, and they were milky white. All isolated strains had the same phenotype characteristics (Table 2).

No β-hemolysis or α-hemolysis, no motility, no nitrate reduction, and no H_2S production were observed. The strains also could not use β-galactosidase, glucose, Simmons citrate, maltose, sucrose, xylose, urea, sorbose, rhamnose, lactose, melibiose, acetamide, mannose, cellobiose, and raffinose, and they could not degrade aesculin.

3.3. Identification of Isolated Strains Based on 16S rRNA and rpoB Sequences. The 16S rRNA sequences of 10 isolated strains were approximately 1400 base pairs (bp). All isolated strains showed a high degree of phylogenetic relationship with *A. johnsonii* (percent identity >99%). According to the phylogenetic tree of 16S rRNA sequences, with *Bacillus thuringiensis* as the outgroup, all isolated strains and *A. johnsonii* (accession nos.: NR_164627, KY118919, and ON237942) clustered into one branch (bootstrap 96.6). Additionally, the strains Ajo2017E-4 and Ajo2017E-7 isolated from eyes were clustered into one subbranch (bootstrap 90.4, Figure 2(a)).

The rpoB sequences of selected strains Ajo2017E-1, Ajo2017E-2, Ajo2017L-9, and Ajo2017L-10 were 356, 363, 344, and 356bp, respectively. The phylogenetic tree results were similar to those of 16S rRNA clustering. Ajo2017E-1, Ajo2017E-2, Ajo2017L-9, Ajo2017L-10, and *A. johnsonii* (accession no.: KU96161) clustered into one branch (bootstrap 98.2, Figure 2(b)). Additionally, different isolation sources from eyes and livers were clustered into two subbranches. Ajo2017E-1 and Ajo2017E-2 strains clustered into one subbranch with bootstrap 95.8, and Ajo2017L-9 and Ajo2017L-10 strains clustered into another subbranch with bootstrap 88.4.

Based on the morphological, physiological, and biochemical data and the 16S rRNA and rpoB sequences of the colonies, the isolated strains were identified as *A. johnsonii.*

3.4. Drug Sensitivity. The strains were susceptible to neomycin sulfate, norfloxacin, and oxolinic acid, and the MIC was 6.25, 0.39, and 0.39 of selected strains Ajo2017L-10. But the strains were resistant to ampicillin sodium salt, doxycycline, thiamphenicol, and sulfamethazine (Table 3). The MIC for these drugs was more than 50 mg/L.

Table 2: The phenotypic characteristics of isolated strains from rainbow trout.

Biochemical tests	Isolated strains	*A. johnsonii* [15]	*A. baumannii* [6]
Gram stain	−	−	−
α-hemolysis	−	−	n.a
β-hemolysis	−	−	n.a
β-galactosidase	−	−	−
Arginine dihydrolase	−	−	−
Ornithine decarboxylase	−	n.a	−
Lysine decarboxylase	−	n.a	−
Peptone solution (0–3% NaCl)	+	n.a	n.a
Peptone solution (6–10% NaCl)	−	n.a	n.a
H_2S production	−	n.a	−
Nitrate reduction	−	−	n.a
Citrate utilization	−	−	−
Aesculin utilization	−	−	n.a
Urease	−	−	−
Gelatinase	−	−	−
Acetamide utilization	−	n.a	n.a
Fermentation/oxidation			
Glucose	−	−	+
Maltose	−	−	−
Sucrose	−	n.a	n.a
Xylose	−	n.a	+
Sorbose	−	n.a	n.a
Rhamnose	−	n.a	−
Lactose	−	n.a	+
Melibiose	−	n.a	+
Mannose	−	−	n.a
Cellobiose	−	n.a	n.a
Raffinose	−	n.a	−

Note: "+": positive; "−": negative; "n.a.": not applicable. This test was not performed for the reference strain.

3.5. Experimental Infection. There were no mortalities during the acclimatization period prior to the infectivity experiment. There was no death in the control injected with 0.65% NaCl during the experimental process. Fifty percent mortality occurred under the 2.5×10^7 CFU/mL treatment with Ajo2017L-10, whereas 90% mortality occurred under the 2.5×10^9 CFU/mL treatment with Ajo2017L-10. The first incidence of mortality was observed 3 days after injection under the 2.5×10^6, 2.5×10^7, and 2.5×10^9 CFU/mL treatment, and all mortality occurred during the first 9 days of the experiment (Figure 3). The affected fish in the treatment groups had the same external appearance as the diseased fish sampled from the fish farm. The bacteria isolated from the liver, head kidney, and spleen of fish in the treatment groups (including dead fish and surviving fish) were identified as *A. johnsonii*.

3.6. Histopathological Changes of Rainbow Trout. Obvious histopathological changes were observed in the liver, head kidney, and spleen of 5 fish in the treatment groups. The liver and spleen in the treatment groups contained a high incidence of vacuolar degeneration (Figures 4(d) and 4(f)), whereas no lesions were detected in the liver and spleen of the fish in the control group. In the head kidney, a large amount of cytoplasm was observed in lymphocytes, and melanin had also accumulated in the lymphocytes (Figure 4(e)).

3.7. Characterization of OMP Family Members. OMP family members including Omp34, OmpA, and OmpTsx were segregated into different branches (bootstrap 100). Omp34, OmpA, and OmpTsx of *A. johnsonii* clustered with *A. baumannii* with a bootstrap value of 95.9, 70.7, and 65.1, respectively (Figure 5).

Predicted protein structures of Omp34, OmpA, and OmpTsx of *A. johnsonii* were constructed by AlphaFold 2. The pLDDT score was used for the assessment of structure prediction (Figure 6). Among the 5 models for each protein, the highest pLDDT score was 75.05 (model 3), 92.59 (model 5), and 94.05 (model 5) for OmpA, Omp34, and OmpTsx, respectively. The model of the OMP family member with the highest pLDDT score was used for 3D protein structure construction. In the protein structure, OmpA included 6 helixes and 5 strands; Omp34 included 5 helixes and 16 strands; and OmpTsx included 3 helixes and 12 strands.

The average scores of hydrophilicity, flexibility, and surface accessibility prediction were 2.433, 1.013, and 1.000 for OmpA, 1.900, 0.994 and 1.000 for Omp34, and 1.408, 0.992, and 1.000 for OmpTsx, respectively. The average antigenic propensity for this protein was 1.0122, 1.0178, and 1.0139 for OmpA, Omp34, and OmpTsx, respectively. There are 8, 10, and 10 antigenic determinants in OmpA, Omp34, and OmpTsx, respectively (Table 4 and Figure 6). OmpA contains 3 antigenic

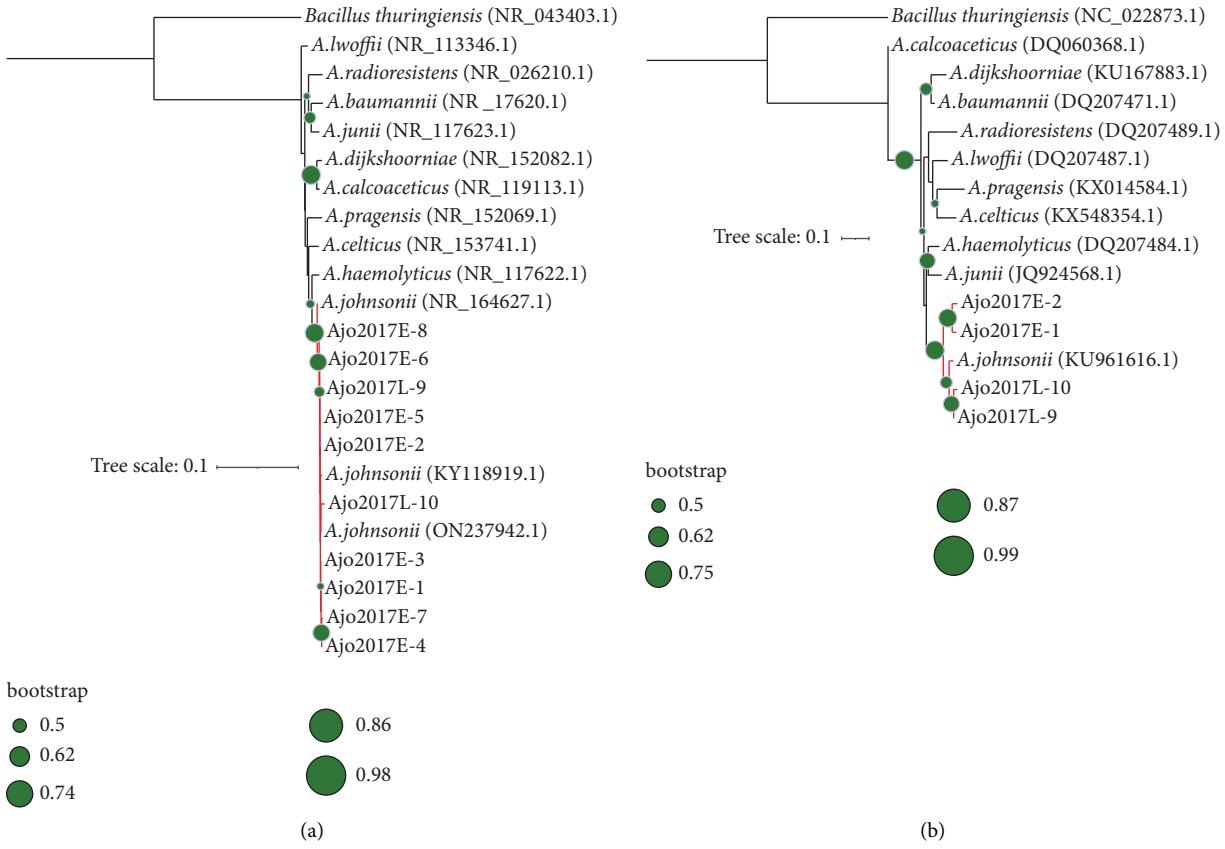

Figure 2: (a) Construction of the 16S rRNA phylogenetic tree of 12 *Acinetobacter* strains by NJ. (b) Construction of the rpoB phylogenetic tree of 10 *Acinetobacter* strains by NJ. The degree of confidence for each branch point was determined by bootstrap analysis (1,000 repetitions). Only bootstrap values >50 are shown.

Table 3: MIC values of the Ajo2017E-1, Ajo2017E-2, Ajo2017L-9, and Ajo2017L-10 strains.

Antimicrobial agents	MIC values (mg/L)			
	Ajo2017L-9	Ajo2017L-10	Ajo2017E-1	Ajo2017E-2
Ampicillin sodium salt	>50 (R)	>50 (R)	>50 (R)	>50 (R)
Neomycin sulfate	6.25 (S)	6.25 (S)	6.25 (S)	6.25 (S)
Ciprofloxacin	0.39 (I)	0.39 (I)	0.39 (I)	0.39 (I)
Doxycycline	>250 (R)	>250 (R)	>250 (R)	>250 (R)
Norfloxacin	0.39 (S)	0.39 (S)	0.39 (S)	0.39 (S)
Florfenicol	6.25 (I)	6.25 (I)	6.25 (I)	6.25 (I)
Sulfadiazine	>25 (R)	>25 (R)	>25 (R)	>25 (R)
Oxolinic acid	0.20 (S)	0.39 (I)	0.20 (S)	0.20 (S)
Thiamphenicol	>50 (R)	>50 (R)	>50 (R)	>50 (R)
Sulfamethazine	>50 (R)	>50 (R)	>50 (R)	>50 (R)

S = susceptible; I = intermediate; R = resistant.

determinants, which are 90% similar with epitopes of *A. baumannii*. Overall prediction for the protective antigen was 0.7523, 0.7580, and 0.7820 for OmpA, Omp34, and OmpTsk, respectively.

3.8. OmpA Expression. OmpA was 654bp long and contained an open reading frame encoding a protein of 217 amino acids, including 22.176 kDa of molecular weight and 9.363 of the isoelectric point. The nucleotide and amino acid sequences showed 84.45% and 100% identity with Acinetobacter spp. (accession numbers CP043909 and MBP9786511) by BLASTN and BLASTP (NCBI web servers), respectively.

After optimization of the flask induction expression, the recombinant protein expressed by the plasmid pET32a-OmpA was nearly 35 kDa compared with the pET32a vector (Figure 7(a)). A large number of expressed proteins of the pET32a vector were almost removed after purification by nickel affinity chromatography. The molecular weight of recombinant protein OmpA was nearly 35 kDa (Figure 7(b)).

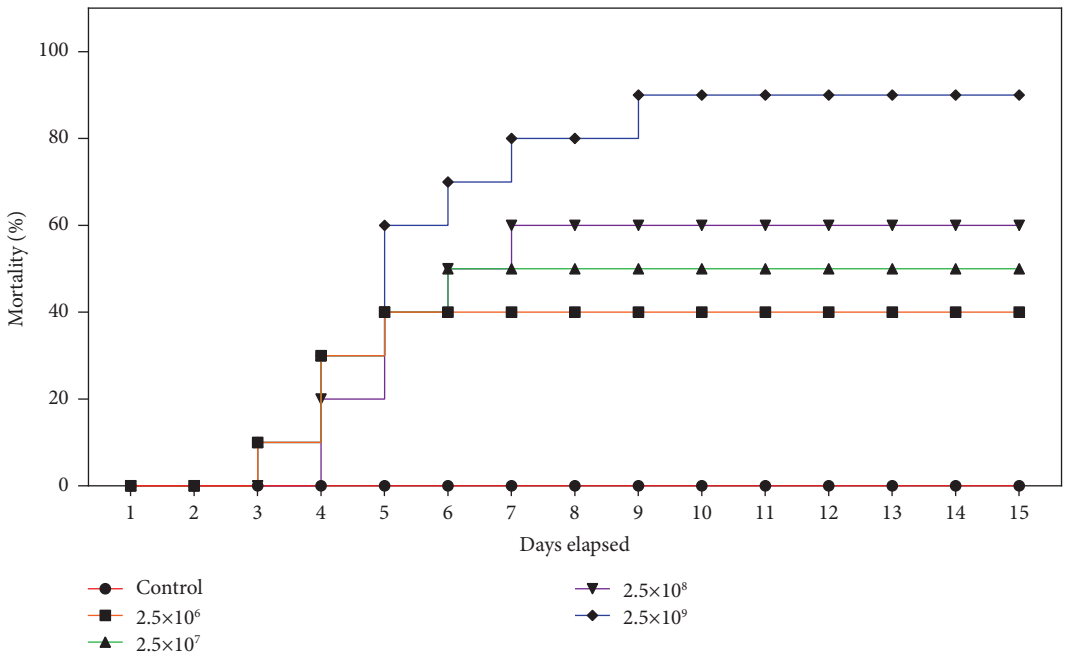

Figure 3: Cumulative mortality curves in rainbow trout challenged with *Acinetobacter johnsonii* (Ajo2017L-10) by intraperitoneal injection at different concentrations. The control group with 0.65% NaCl solution.

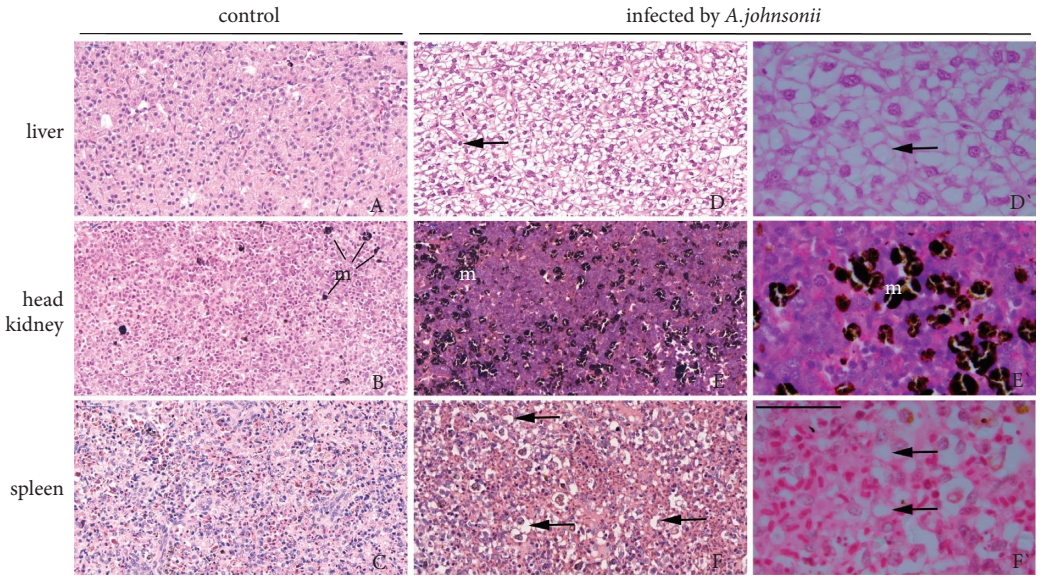

Figure 4: Histological analysis of the liver, head kidney, and spleen of normal rainbow trout and rainbow trout infected by *A. johnsonii*. Arrows indicate a high degree of vacuolar degeneration. m: melanin. Scale bar = 50 μm.

4. Discussion

Acinetobacter is widely distributed in the external environment and can be isolated in water, soil, humans, and animals [2], but it is generally considered to be rarely pathogenic [32, 33]. The species of *Acinetobacter* can cause infectious diseases, which is known as a typical opportunistic pathogen in fishes [34, 35]. The damage that *Acinetobacter* causes to fish displayed symptoms such as ascites and ulceration on the entire body resembling the septicaemic form of infection [9, 36]. Different bacteria species of genus *Acinetobacter* such as *A. lwoffii*, *A. junii*, *A. baumannii,* and *A. pittii* were isolated from diseased pig frogs (*Rana grylio*) with necrotic skin [37], stone flounder (*Kareius bicoloratus*) with bacterial septicemia [38], channel catfish (*Ictalurus punctatus*) [6], and pond loach (*Misgurnus anguillicaudatus*) [8], respectively. However, there are few studies that have focused on *A. johnsonii*. Therefore, *Acinetobacter* spp. are emerging pathogens for fish, as they are widely present in various aquaculture animals and may cause diseases in such systems.

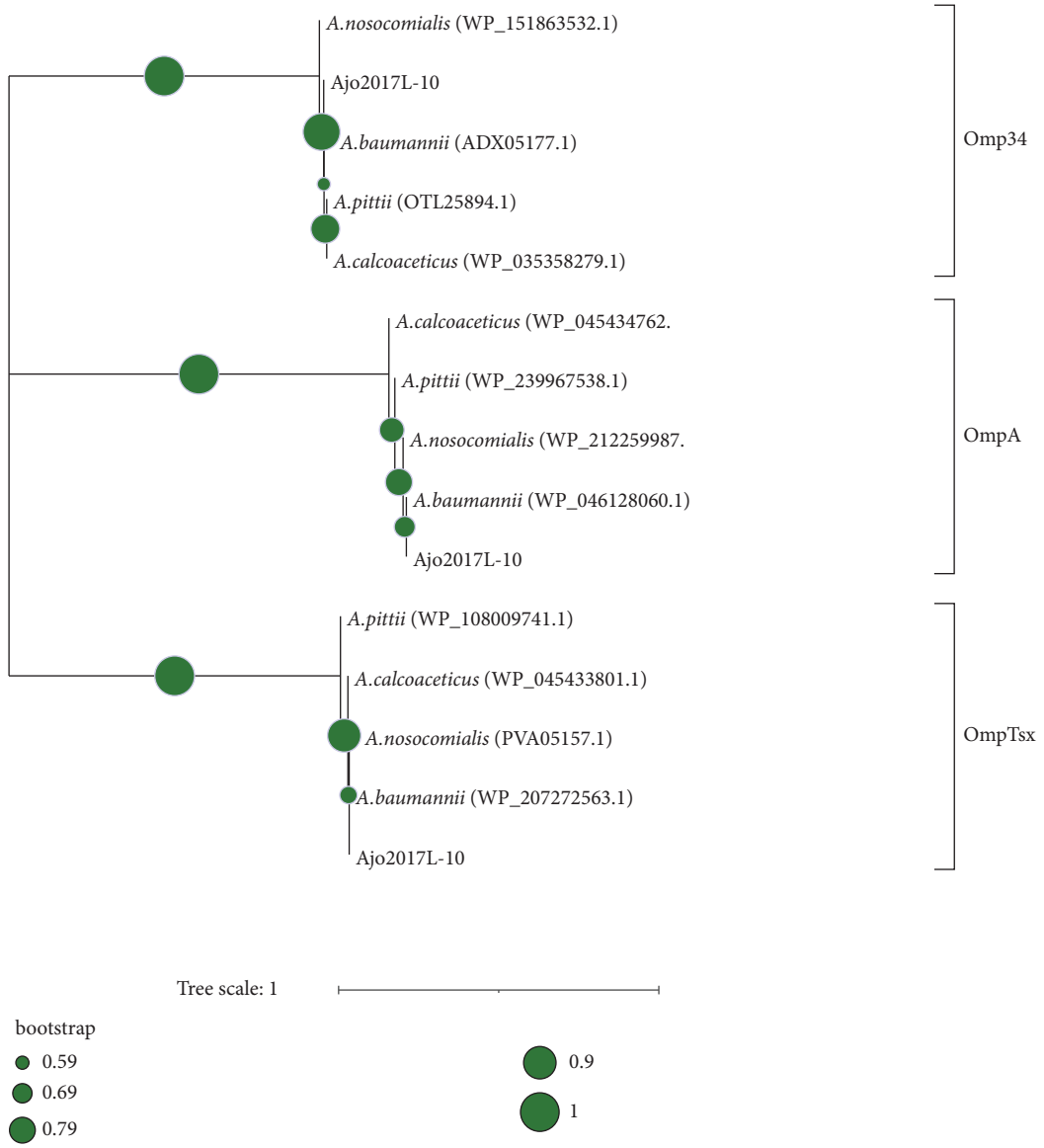

Figure 5: Construction of the Omp34, OmpA, and OmpTsx phylogenetic tree of 5 *Acinetobacter* strains by NJ. The degree of confidence for each branch point was determined by bootstrap analysis (1,000 repetitions). Only bootstrap values >50 are shown.

Rainbow trout were introduced for aquaculture in China in 1959, which is an important fishing resource in Yunnan Province [39]. With increasing cultured density, bacterial diseases have occurred more frequently in recent years. Many of the phenotypic characteristics of the isolated strains in the present study are common to other *Acinetobacter* species. Due to the high specificity and conservation of 16s rRNA gene sequences, 16S rRNA gene amplification and sequencing are currently used for bacterial identification [40]. However, some species of *Acinetobacter* are very closely related and display similar phenotypic and biochemical properties [41]. Differentiating *Acinetobacter* at the species level using 16S rRNA sequencing is not effective due to the extremely low polymorphic nature of the variable region [42]. The resolution of the housekeeping gene rpoB is significantly higher than that of the 16S rRNA gene for bacterial identification [43, 44]. Therefore, combined rpoB and 16S rRNA gene sequencing can be used to improve the accuracy of the identification of *Acinetobacter* species [44]. In this study, 16S rRNA and rpoB sequencing showed that 10 isolated strains are *A. johnsonii*. The infectivity experiment illustrated that these strains cause mortality in rainbow trout. *A. johnsonii* was also reported to be an emerging opportunistic pathogen in farmed rainbow trout [15]. However, to date, *Acinetobacter* has not caused a huge number of rainbow trout deaths. Nonetheless, the identification of *A. johnsonii* as a virulent pathogen of rainbow trout in Yunnan freshwater fisheries indicates that it is an emerging pathogen in this setting.

As for the histopathological characteristics, exposure to *A. johnsonii* resulted in obvious lesions in the liver, spleen, and head kidneys of the infected fish. It could be inferred that the liver, spleen, and head kidney are the major target

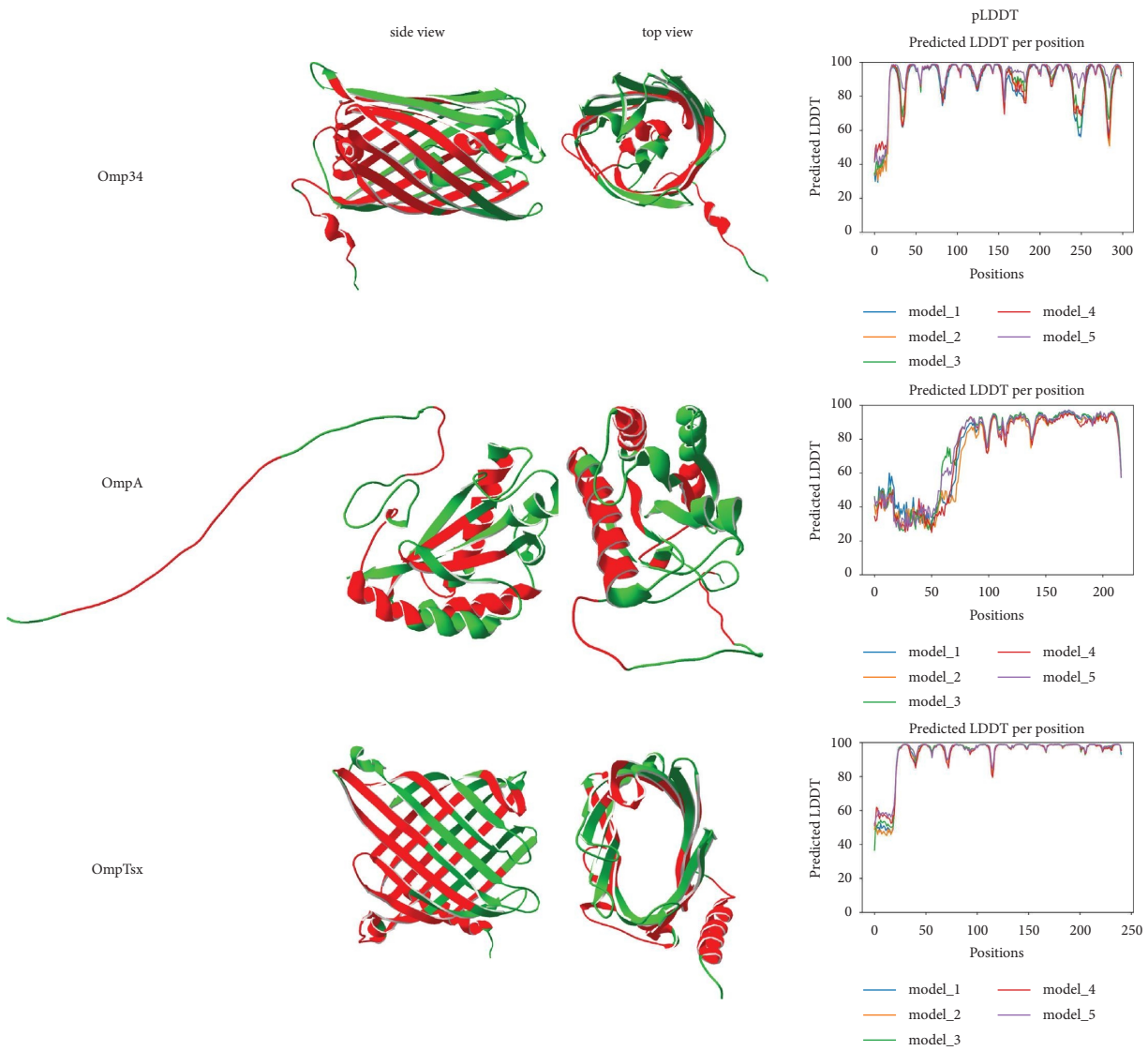

Figure 6: Predicted protein structures of deduced amino acid sequences of Omp34, OmpA, and OmpTsx of *A. johnsonii* by AlphaFold 2. The predicted local distance difference test (pLDDT) was used for the assessment of structure prediction. The red labeled peptides indicate predicted antigenic determinants (shown in Table 4) using the method of Kolaskar and Tongaonkar [31].

organs of *A. johnsonii* in rainbow trout, which has also been reported for *A. baumannii* and *A. pittii* [6, 8]. In the head kidney, a large volume of cytoplasm was observed in lymphocytes, which suggests the activation of B lymphocytes that may result in the synthesis and secretion of antibodies [45, 46]. Macrophages contain heterogeneous inclusions, including melanin, hemosiderin, and lipofuscin [47]. These inclusions accumulated in the head kidneys of diseased rainbow trout in this study. However, the mechanism underlying the accumulation of inclusions requires further study.

A. baumannii was reported to be a multidrug-resistant species in hospitals [48]. When the concentrations of *A. baumannii* are above 10^3 CFU/mL in the surrounding water, freshwater fish from the natural environment are probably colonized for the spread of extensively drug-resistant bacteria [49]. In this study, the isolated strains were resistant to ampicillin, sulfadiazine, thiamphenicol, doxycycline, and sulfamethazine. These antibiotics had never been used in the fish farms before, suggesting that the isolated strains probably originated from contamination by humans with bacteriaemia [15]. Thus, clinical strains may transfer drug-resistant genes horizontally into aquatic microorganisms, causing fish disease, and as a result, they may have a great impact on drug resistance transfer in aquaculture. The vaccine is a safe and effective method to prevent and treat diseases; however, vaccines based on inactivated whole strains can elicit antibodies against multiple surface proteins [50]. OmpA, a major component of OMPs in Gram-negative bacteria, is a predominant antigen in enterobacterial outer membranes [51]. Omp34 is a virulence factor associated with the adherence and invasion of mammal epithelial cells [52]. It has been reported that OmpA-DNA and recombinant

Table 4: The antigenic determinants in OmpA, Omp34, and OmpTsx of *A. johnsonii*.

	Positions	Sequences
OmpA	4–19	LVISTVVGAAVVLSGC
	35–42	ALGTLIGA
	63–78	AAIGAVLGAAGGLYLD
	101–107	GSVQLIM*
	124–134	FYATLDKVAQT*
	140–146	KSAILVT
	157–171	INIPLSQARAQSVKN*
	174–181	AGKGVPSS
Omp34	4–14	LGLATAVLLAM
	16–32	GAHAYQFEVQGQSEYVD
	57–80	KGPLAEAAFLNQASSVSLGYSYQQ
	86–93	VNYHIGTY
	95–112	VKGEAYVPTPYLPVYASA
	134–158	YALEVGAMLLPNFLMTVGYTSVANQ
	171–177	IYSAVNQ
	186–197	DAVTARAKYVGP
	220–235	GLKTDLYLTPKLSVGA
	262–274	VNYFITPALAVGA
OmpTsx	4–22	KQLAATCALLSATAMVQAK
	24–32	IWQDFSVTG
	36–42	ENYEVVD
	48–63	ITLEYAAKVKYADVFF
	75–89	KSTYFELSPRLSLGE
	91–105	SGKKLAYGPIKDVLI
	120–139	DNFLYGFAVDLDIPYFQYAN
	155–165	QMTFVYGIPFK
	168–175	SEDFLVDG
	206–216	PDTRLYLGIEH

*OmpA contains 3 antigenic determinants, which are 90% similar with epitopes of *A. baumannii*.

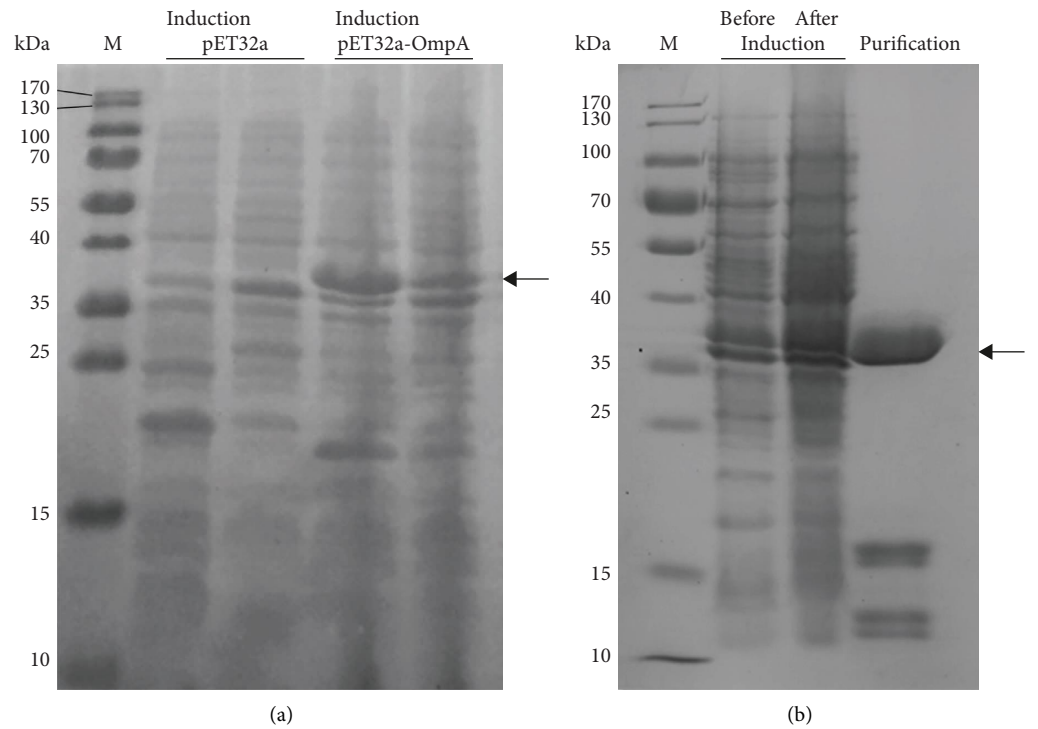

Figure 7: OmpA expression in prokaryocytes. (a) Recombinant plasmid pET-32a-OmpA and its expression. (b) Expression and purification of recombinant plasmid pET-32a-OmpA.

Omp34 vaccine are potent enough to trigger humoral and cellular immune responses to protect mice against *A. baumannii* infection [16, 53, 54]. Ion channel protein Tsx is a nucleoside-specific integral OMP that functions as a substrate-specific channel for deoxynucleosides [55]. Importantly, Tsx has been related to resistance to tetracycline and ampicillin [56]. Therefore, in this study, more resistance to ampicillin and doxycycline of the isolated strains may be linked to OmpTsx.

AlphaFold is a novel machine learning approach that incorporates the evolutionary, physical, and geometric constraints of protein structures, greatly improving the accuracy of protein structure prediction [57]. In this study, the structure of Omp34 and OmpTsx of *A. johnsonii* forms a monomeric, β-barrel porins with a long and narrow channel spanning the outer membrane, which was similar to other studies [17, 58]. B-barrels begin to fold into a β-sheet-like structure before they are integrated into the outer membrane of Gram-negative bacteria [59]. The pLDDT score of OmpA was lower than that of Omp34 and OmpTsx; the structure of OmpA was not similar to the membrane topology of the N-terminal domain of OmpA [51]. It is expected that a low-confidence prediction will be produced by AlphaFold, and regions with pLDDT <50 that are intrinsically disordered or unstructured will have a ribbon-like appearance [60]. The OMPs family forms β-barrel with a long and narrow hydrophobic channel, involved in the transport of small hydrophobic molecules across the bacterial outer membrane [61]. *A. baumannii* secretes cytotoxic OmpA to host cells via outer membrane vesicles, which may contribute to pathogenesis during *Acinetobacter* infection [62].

In conclusion, based on the morphological, physiological, and biochemical characteristics and the 16S rRNA and rpoB sequences of the strains isolated from diseased rainbow trout, the isolated strains were preliminary identified as *A. johnsonii*. The isolated strains were highly sensitive to florfenicol, ciprofloxacin, and norfloxacin but resistant to ampicillin, sulfadiazine, thiamphenicol, doxycycline, and sulfamethazine. According to the characterization of OMP family member analysis, OmpA protein could be used as a subunit vaccine candidate molecule of *A. johnsonii*. Further research is needed to be performed on the protection rate of OmpA subunit vaccine against *A. johnsonii*.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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