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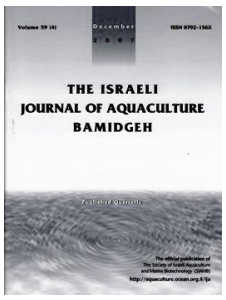

Isolation and Characterization of *Acinetobacter lwoffii* from the Intestine of Grass Carp (*Ctenopharyngodon idella*)

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Keywords: grass carp; *Acinetobacter lwoffii*; isolation; identification; pathogenicity

Abstract

Acinetobacter lwoffii infections are largely concentrated in mammals; very few reports are available for such infections in fish. In this study, a Gram-negative rod bacillus was isolated from the intestine of grass carp (*Ctenopharyngodon idella*), and designed as strain CY1. The isolated strain was then identified as *A. lwoffii* according to morphological, physiological, and biochemical properties, and 16S rDNA sequencing. The phylogenetic tree constructed on the basis of 16S rDNA sequences indicated that strain CY1 is most closely related to *A. lwoffii*, and has 98.08% similarity with type strain ATCC17925. Experimental infection assays were conducted, and a cumulative mortality rate of 75% was observed in grass carp. Antimicrobial susceptibility test showed that the isolated strain CY1 was highly susceptible to most antimicrobial agents tested, including aminoglycosides, β-lactams, and quinolones. To our knowledge, this is the first report on pathogenic *A. lwoffii* in grass carp.

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Introduction

Acinetobacter lwoffii is an aerobic, non-fermentative gram-negative bacill (Rathinavelu et al., 2003; Takehiko et al., 2005; Ku et al., 2000), which is non-fastidious and can utilize a wide variety of substrates as their sole energy source (Rathinavelu et al., 2003). Hence, these organisms are generally ubiquitous and are frequently found in soil, water, dry environments, and hospitals (Rathinavelu et al., 2003). Due to its ubiquitous nature, it is a potential opportunistic pathogen in patients with impaired immune systems and it has been identified as a cause of nosocomial infections (Bergogne-Berezin et al., 1996; Forster et al., 1998). It is a well-known pathogen associated with gastritis, septicemia, pneumonia, meningitis, and urinary tract infections in humans (Luciano et al., 2007; Wareham et al., 2008; Sule et al., 1997; Idzenga et al., 2006; Medina et al., 2007; Kim et al., 2014). Infections caused by *A. lwoffii* in previously reported cases have mostly concentrated in humans; few reports are available in fish (Kozińska et al., 2014; Dadar et al., 2016).

Grass carp (*Ctenopharyngodon idella*) is a long slender member of the minnow family native to large rivers in Asia. It is one of the most abundant freshwater fish in China, with production of over 3 million tons per year, which is 20% of the total yield of freshwater fish. However, bacterial disease outbreaks have caused great economic losses in cultured grass carp (Rathinavelu al et al., 2003). Some bacterial pathogens such as *Shigella* sp, *Escherichia coli,* and *Serratia fonticola,* have been isolated from diseased grass carp (Toyoshima et al., 2010), but there have been no reports about *A. lwoffii* isolated from diseased grass carp.

In this study, a Gram-negative bacterium was isolated from the intestine of grass carp, and then identified as *A. lwoffii* by 16S rDNA gene sequences. The pathogenicity was confirmed in fish by challenge infection. The grass carp infected by *A. lwoffii* also showed serious hemorrhages in pectoral, ventral, and anal fins. The first description of isolation and characterization of *A. lwoffii* from grass carp provides a scientific reference for fish disease diagnostics.

Materials and methods

Isolation and culture of the bacterial pathogen from grass carp. Bacterial pathogens were isolated from intestinal samples of seemingly healthy grass carp according to a previous method (Hu et al., 2010). The grass carp were collected from a commercial fish farm at Xuzhou City, in China. Samples were streaked onto Luria-Bertani (LB) agar plates supplemented with tryptone (10 g/L), yeast extract (5 g/L), and NaCl (5 g/L) incubated at 28^{0}C for 18-24 h. Single colonies from plates were re-streaked on the same media to obtain pure isolates. The isolates were stored at -80^{0}C in LB broth at a final concentration of 15% glycerol.

Physiological and biochemical test. Morphological investigation was conducted using the gram-staining method. Biochemical tests were performed using a micro-bacteria biochemical test system (Tianhe Microbial Reagent Co., Ltd, China), including lysine decarboxylase, lysine decarboxylase, oxidase, arginine dihydrolase, and urease; arginine dihydrolase as well as urease; nitrate reductase, gluconate and DNase; reactions for Methyl red and Voges-Proskauer; acid production from sorbitol and erythritol, lactose, arabinose, raffinose, sorbose, etc. The micro-bacteria biochemical test tube was incubated in an incubator at 28^{0}C for 48h.

Antimicrobial susceptibility test. The antimicrobial susceptibility tests of the isolate were conducted using Kirby-Bauer disc diffusion method on Mueller-Hinton agar plates, with results interpreted according to the guidelines of the Clinical and Laboratory Standards Institute. Each value is a mean of three replicates. The antimicrobial agents (Tianhe Microbial Reagent Co., Ltd, China) included were [The number in brackets indicates the dose of each antibiotic (μg per disc)]: ampicillin (10 units), amoxicillin (10), carbenicillin (100), meropenem (10), imipenem (10), cefamandole (30), cefixime (5), cefotaxime (30), cephalothin (30), cephalexin (30), cefoperazone (75), piperacillin (100), amikacin (30), gentamicin (10), kanamycin (30), netilmicin (30), neomycin (30), streptomycin (10), tetracycline (30), chloramphenicol (30), nitrofurantoin (300), norfloxacin (10), ofloxacin(5),pefloxacin(10), enrofloxacin(5), enoxacin(10),

sulfamethoxazole/trimethoprim (23.75/1.25), sulphafurazole (300), trimethoprim (5), azithromycin (15), erythromycin (15), teicoplanin (30), vancomycin (30), rifampicin (5), clindamycin (2), lincomycin (2).

16S rDNA gene sequences analysis. Total genomic DNA of the isolate strains was extracted with a column genomic DNA extraction kit according to the manufacturer's protocol. Oligonucleotide primers were used to amplify a 16S rDNA amplicon of approximately 1,500 bp. The primer sequences were generated according to the method of Vaneechoutte et al (Lü et al., 2016), and were as follows: 5'- AGAGTTTGATGGCTCAG -3' (forward primer) and 5'- AAGTCGTAACAAGGTAACCGTA -3' (reverse primer). PCR amplification conditions were as following: an initial denaturation step at 95^0C for 2min; 30 cycles of 95^0C for 30s, annealing for 30s and 72^0C for 1min; a final extension step at 72^0C for 10 min. PCR amplification was carried out in 25μL volumes of incubation buffer containing 1 μL of bacterium solution, 1 μL×2 of 27F/1492R, 12.5 μL of Master Mix, 9.5 μL of ddH_2O. The PCR product was analyzed by gel electrophoresis.

Experimental infections. Sixteen healthy grass carp were fed with commercial feed (Tongwei Co., Ltd, China) for the duration of the seven day infection experiment. The grass carp were divided into two groups with eight grass carp in each group. Fish were infected by intraperitoneal (i.p.) injection with 0.2 ml dosage of approximately 3.83×10^8 CFU/ml. The control group was treated with 0.2 mL of 0.65% normal saline. The temperature of the water was about 20^0C throughout this test.

Results

Morphology and biochemical characteristics of CY1 strain. One bacteria strain was isolated from grass carp intestines and tentatively named strain CY1. Through isolation and purification, the colonies of CY1 on LB plates looked circular, semitransparent, smooth, humid, and ridgy as shown in Fig. 1 (A). The bacterial cells were Gram-negative rod-shaped as shown in Fig. 1 (B). Biochemical tests results are summarized in Table 1, the results showed that CY1 was positive for xylose, fructose, xylitol, while was negative for gelatin, power, oxidase, etc. CY1 is weakly positive for arginine dihydrolase.

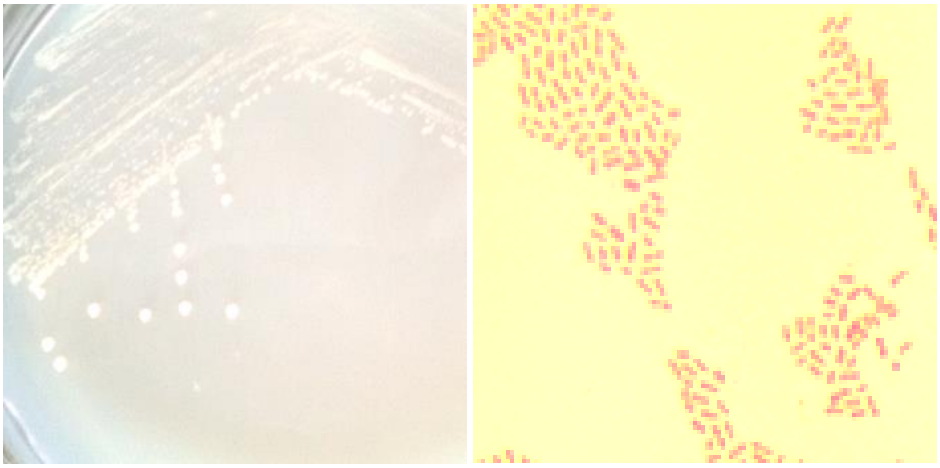

Fig. 1. A) The colonies of CY1 on LB plates looked circular, semitransparent, smooth, humid and ridgy. B) *A. lwoffii* form Gram-negative rod-shaped clusters.

Table 1. Morphological and biochemical properties of the strain CY1 isolated from grass carp.

Characteristics	*CY1*	*Characteristics*	*CY1*
Fructose	+	Glucose (gas)	−
Motility	−	Xylitol	+
Melibiose	−	Sorbitol	−
Oxidase	−	Raffinose	−
Lactose	−	Xylose	+
Arabinose	−	Mannose	−
Ornithine decarboxylase	−	Phenylalanine deaminase	−
Sucrose	−	Maltose	−
Lysine decarboxylase	−	Nitrate reductase	−
Glucose	−	Erythritol	−
Arginine dihydrolase	±	Mannitol	−
Urease	−	Adonital	−
N-acetylglucosamine	−	Inositol	−
Nitrate reduction	−	MR	−
H_2S	−	Gluconate	−
VP	−	Citrate	−

Note: +: positive. −: negative. ±: weak reaction.

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGA
GCGGGGAAAAGTAGCTTGCTACTTAACTTAGCGGCGGACGGGTGAGTAATACTTAGGA
ATCTACCTAATAGTGGGGGACAACATCTCGAAAGGGATGCTAATACCGCATACGCCCTA
CGGGGGAAAGCAGGGGACTCTTCGGAGCCTTGCGCTAATAGATGAGCCTAAGTCGGAT
TAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGG
ATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG
CCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTCTAGTTAATACCTAGAGA
TAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAA
TACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGTGGCCA
ATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTGG
CTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGGAGGAATACCGATGGCGAAGGCAGCCATCTGGGCCTAATACTGACACTGAGGTACG
AAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTC
TACTAGCCGTTGGGGTCTTTGAGACTTTAGTGGCGCAGCTAACGCGATAAGTAGACCG
CCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACAT
AGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAGCTTACATACAGGTGCTGCAT
GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTTTCCTTATTTGCCAGCGGGTTAAGCCGGGAACTTTAAGGATACTGCCAGTGACAAAC
TGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACA
CGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAA
AGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTA
GTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTGCACACCGCCC
GTCACACCATGGGAGTTTGTTGCACCAGTAGTAGGTAGTCTAACCGCAAGGAGGACGC
TTACCACGGTGTGGCCGATGACTGGGGTGAAGTCGTAACAAGGTAACCGTA↵

Fig. 2. A) PCR amplification of 16S rDNA gel electrophoresis of strain CY1. Line 1 is DNA marker, line 2 is 16S rDNA. B) 16S rDNA fragment size is 1505 bp, and the primers are the red sequences.

16S rDNA gene sequences analysis. The 16S rDNA gene sequences of the isolate CY1 was approximately 1500 bp in length (Fig. 2A), and demonstrated a restriction fragment pattern identical to that of DNA extracted from the original *A. lwoffii* ATCC17925. Figure 2B shows the PCR products of the expected 1505 bp 16S rDNA fragment size of the CY1 strain. The results of Blast alignments showed that the isolate CY1 shared the highest 16S rDNA sequence identities (98.08%) with *A. Lwoffii* type

strain ATCC17925（HE651921). The phylogenetic tree based on 16S rDNA sequences showed that the isolate CY1 is most closely related to the new strains *A. lwoffii* ATCC 17925（Fig.3).

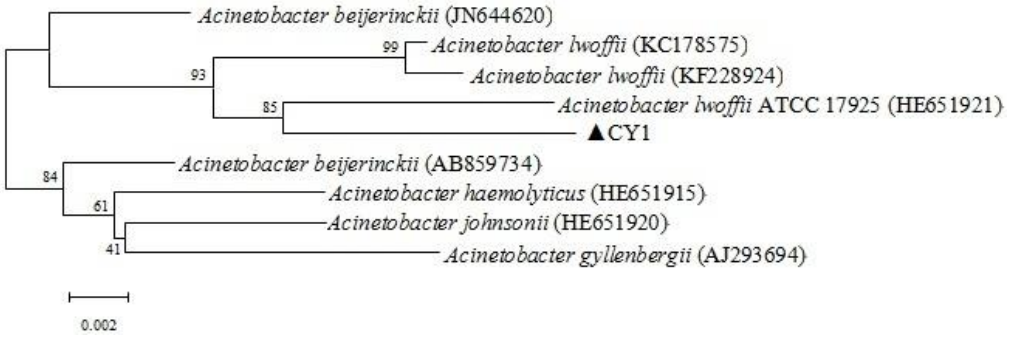

Fig. 3. Phylogenetic tree analysis of Acinetobacter species based on 16S rDNA nucleotide sequences. Unrooted tree was generated using neighbor-joining method by the MEGA4.1 software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. The scale bar represents 0.002 substitutions per site. Bootstrap values above 45% are shown at the nodes. GenBank accession numbers of type strains are given in brackets, and the isolate CY1 identified in this study is indicated by the shaded triangle.

Experimental infection results. Following the infection with strain CY1, mortality rate was 75%. The dead grass carp exhibited hemorrhages on the pectoral, ventral, and anal fins, and on their bodies.

Antimicrobial susceptibility test. The results of antimicrobial susceptibility tests showed that the isolate CY1 strain had high susceptibility to most of the tested antimicrobial agents, including aminoglycosides, β-lactams (penicillins and cephalosporins), quinolones (except norfloxacin) and cephalosporin (except cefixime); intermediate susceptibility to sulphonamides, cefixime, norfloxacin and erythrocin; low susceptibility to clindamycin, vancomycin, teicoplanin and trimethoprim (Table 2).

Table 2. Antimicrobial sensitivity test of CY1 strain

Antimicrobial	*Concentration (μg/piece)*	*inhibition zone diameter CY1*	*Antimicrobial*	*Concentration (μg/piece)*	*inhibition zone diameter CY1*
Amoxicillin	10	28H	Gentamicin	10	25H
Piperacillin	100	23H	Kanamycin	30	28H
Ampicillin	10	29H	Netilmicin	30	30H
Cefoperazone	75	17H	Sulfafurazole	300	14M
Cefotaxime	30	20H	Cotrimoxazole	23.75/1.25	18H
Cefalotin	30	7N	Trimethoprim	5	N
Cefamandole	30	18H	Vancomycin	30	7N
Cefixime	5	14M	Teicoplanin	30	N
CephalosporinIV	30	18H	Tetracycline	30	30H
Erythrocin	15	12M	Chloromycetin	30	36H
Azithromycin	15	9N	Nitrofurantoin	300	14M
Norfloxacin	10	11M	Rifampicin	5	23H
Nalidixic acid	30	24H	Metronidazole	5	N
Ofloxacin	5	24H	Nystain	100	N
Clidamycin	2	N	Enrofloxacin	5	36H
Neomycin	30	32H	Levofloxacin	5	36H
Imipenem	10	37H	Meropenem	10	35H
Streptomycin	10	31H	Enoxacin	10	30H
Pefloxacin	10	35H			

H: inhibition zone diameter > 15 mm. M: 10 mm≦ inhibition zone diameter ≦ 14 mm. N: inhibition zone diameter<10 mm

Discussion

Numerous published studies focusing on the diversity of *Acinetobacter* spp. refer to the potential of some members of this genus to act as opportunistic pathogens in higher vertebrates, and development of antibiotic resistance (Ku et al., 2000; Joly-Guillou et al., 2005). *Acinetobacter baumannii, Acinetobacter johnsonii*, and *A. lwoffii*, have emerged as important nosocomial pathogens because of their persistence in the hospital environment and broad antimicrobial drug resistance patterns (Bergogne-Berezin et al., 1996; Murray et al., 2005). They are often associated with clinical illness including bacteremia, pneumonia, meningitis, peritonitis, endocarditis, and infections of the urinary tract and skin (Rathinavelu al et al., 2003; Takehiko et al., 2005; Ku et al., 2000). One of the main reasons for disease caused by *Acinetobacter* is, in part, due to their capability to form biofilms (Idzenga et al., 2006; Medina et al., 2007; Henwood et al., 2002). Another reason is the fact that clinical isolates of these bacteria are frequently resistant to the major antibiotics used to treat nosocomial infection (Henwood et al., 2002; Murray et al., 2005; Srinivasan et al., 2009; Towner et al., 1997; Zhang et al., 2013).

Although *Acinetobacter* spp. can also cause infections in freshwater fish, to our knowledge, the diversity and dynamics of members of this genus in grass carp has not been addressed in literature (Towner et al., 2006; Carlos et al., 2013; Dadar et al., 2016). Some studies only characterize the bacterial community; they do not report the lethality of *Acinetobacter* spp. in freshwater fish (Gonzalez et al., 2000). In this study, cultivable *A. lwoffii* resulted in a mortality rate of 75% at a dose of 3.83×10^8 CFU/mL and was confirmed as a pathogen of grass carp with a severe mortality rate. In this study, *A. lwoffii* strain CY1 showed low susceptibility to clidamycin, trimethoprim, and teicoplanin. However, it showed high susceptibility to aminoglycosides, β-lactams, and quinolones, which provides information for clinical treatment and infection prevention caused by *A. lwoffii* in fish. This new finding in fish is of concern, as the use of antibiotics may disrupt the normal flora and facilitate the emergence of opportunistic infection. Understanding *Acinetobacter* virulence factors is still at an elementary stage. However, the ability of *Acinetobacter* spp. to produce extracellular enzymes and toxins, their ability of adherence to epithelial cells, the role of polysaccharidic capsule, and surface components protecting the bacteria from phagocytosis has been reported (Srinivasan et al., 2009; Grziela et al., 2008). The study of *Acinetobacter* virulence factors and correct adaptation of the use of antibiotics capable of eradicating them is imperative.

In summary, this is the first report of *A. lwoffii* from grass carp. Experimental challenges were conducted and demonstrated the pathogenicity of the *A. lwoffii* isolate for grass carp. 16S rDNA gene sequence analysis of isolate CY1 will provide scientific reference for fish disease diagnostics. Further research is needed to investigate the virulence of *A. lwoffii* isolated from farmed fish.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 31272692), Key Scientific Research Project Universities and Colleges in Henan province (No. 17A240001), Natural Science Foundation of Tianjin city (No. 16JCZDJC33500, 15JCZDJC34000), Innovation Team of Tianjin Fisheries Research System (No. ITTFRS2017009) and Team of Provincial Science and Technology Innovation of Henan High Education (No. 15IRTSTHN018).

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