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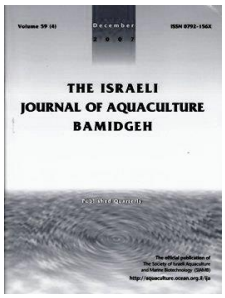

Citrobacter freundii: a Causative Agent for Tail Rot Disease in Freshwater Cultured Japanese Eel *Anguilla japonica*

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Key words: Tail rot disease; *Anguilla japonica*; *Citrobacter freundii*; antibiotic resistance.

Abstract

Tail rot disease causes significant economic damage in freshwater farmed Japanese eel *Anguilla japonica,* yet information on *Citrobacter freundii* as a possible causal agent for this disease is scarce. In this study, a virulent strain, temporarily named MINA, was isolated from diseased *A. japonica* suffering from tail rot disease. It was identified through phylogenetic analysis and phenotypic characteristics and thus compared to other known isolates. Isolate MINA has developed multiple resistances to penicillin, quinolones and sulfonamide antibiotics as well as to amide alcohols, cephalosporin, glycopeptide and macrolide drugs used in aquaculture when screened against a range of common antibiotics. To the best of our knowledge, this is the first report of tail rot disease caused by *C. freundii* in freshwater farmed *A. japonica.*

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Introduction

Japanese eel *Anguilla japonica* are widely cultivated in China, Japan, Korea and Malaysia (Shahkar et al., 2016). *A. japonica* has become the most important and profitable commercial eel species in China (Zhang et al., 2011). Production increased to over 30,000 tons in 2014 (Huang et al., 2015). However, bacterial diseases have become a major economic problem in freshwater farming of *A. japonica* (Joh et al., 2013). More attention must be paid to bacteriosis to enable the development of sustainable freshwater eel farming.

Tail rot disease has caused significant economic losses in the freshwater cultured eel industry (Wang et al., 2001). Several bacterial pathogens such as *Aeromonas punctata* and *Aeromonas sobria* have been reported to cause tail rot disease in freshwater farmed *A. japonica* (Fan et al., 1995; Li and Wei, 2000) however no information is available for *Citrobacter freundii* as a causal agent of tail rot disease in freshwater farmed eels.

In this study, *C. freundii* was isolated from freshwater cultured *A. japonica* suffering from tail rot disease in Shanghai China in June 2015. The aim of this study was to characterize the phenotype, taxonomic position, and antibiotic sensitivity of this strain. As far as we know, this is the first report of involvement of *C.freundii* in tail rot disease in freshwater farmed *A. japonica*.

Materials and methods

2.1 Japanese eel samples. Sixteen diseased freshwater cultured *A. japonica* (av. weight 60.3±1.5 g) suffering from an outbreak of tail rot disease were sampled from an eel farm in Shanghai China during June 2015. The eels were stocked in 1200 square meters of ponds at an initial rearing density of 375 juveniles per square meter. Water quality during the disease outbreak was pH 6.67, 0.40 mg/L total ammonia, 0.12 mg/L^{-}nitrite and 6.12 mg/L^{-}dissolved oxygen. Even though this was the first outbreak of the disease at this farm it could not be controlled by application of potassium permanganate and providone-iodine reported as commonly used effective antibacterial agents (Rico et al., 2012). Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

2.2 Isolation of Bacteria. Each sampled diseased eel was disinfected externally with 75% alcohol and dissected. A 0.1 g section of rotten tail muscle and a liver sample of each eel was cut and streaked onto nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.). After incubation for 24h at 28^{0}C, the dominant uniform isolates were purified by streaking and re-streaking onto NA plates. Pure isolates of the dominant colonies were stored at -80^{0}C supplemented with 15% glycerol. A representative of the dominant isolates, temporarily named MINA, was further characterized in the present study.

2.3 Identification of the pathogen

2.3.1 Molecular identification. The extraction of genomic DNA from isolate MINA, as well as PCR amplification and sequencing of its 16S rRNA gene were performed according to our previous study (Cao et al*.,* 2010). The near complete 16S rRNA gene sequence was assembled using MegAlign, Editseq and Seqman software. A search was performed in the National Centre for Biotechnology Information (NCBI) database for sequence homology using the Basic Local Alignment Search Tool (BLAST) program. A phylogenetic tree from the near complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbor-joining method.

2.3.2 Phenotypic identification. Isolate MINA was identified phenotypically by API 20E system recommended by Topic Popovic et al. (2007) where the isolate MINA was grown on nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.) at 28^{0}C for 24h.The bacterial suspension was then used to inoculate the Analytical Profile Index (API 20E) test strips (Biomerieux, France) following manufacturer's instructions. The plate was incubated at 37^{0}C for 18h and checked against the API identification index and database (Dong and Cai, 2001).

2.4 Bacterial virulence assay. Bacterial virulence was examined by experimentally infecting 100 healthy freshwater cultured eels (av. weight 56.3±2.8 g) obtained from Qinhuang fishery Co., Ltd. in Shanghai China. Their health status was assessed according to guidelines in our previous study (Cao et al., 2013). The eels were acclimated in ten, 60 L aquaria aerated with filtered farming water at 28^{0}C for 14 days (10 fish/aquarium).

Prior to the bacterial virulence assay the isolate MINA was inoculated onto NA plate, incubated at 28^0C for 24h, then washed with normal saline and placed in a sterile tube. Cell density was determined by counting colony forming units after a ten-fold serial dilution in sterile distilled water. Two replicates of 10 healthy fish were inoculated with 0.1 mL of the isolate MINA at a concentration of 2.0×10^5 CFU/mL to 2.0×10^8 CFU/mL injected intramuscularly into the tail. Two more replicates of 10 healthy fish exposed to the same experimental conditions and injected intramuscularly with 0.1 mL of normal saline served as control. The experimental fish were kept at 28^0C and observed daily for seven days without feeding or water change. Any dead eels were immediately removed and sampled to reisolate and confirm if mortality was caused specifically by the inoculated isolate. The mean lethal dose (LD_{50}) value was calculated using the linear regression method recommended by Won and Park (2008).

2.5 Antibiotic sensitivity assay. Antibiotic sensitivity of isolate MINA was assayed on NA plates using the Kirby-Bauer disk diffusion method recommended by Jones et al. (2001). Twenty-six fishery antibiotic discs were acquired from Hangzhou Tianhe Microorganism Reagent Co., Ltd. The zones of inhibition were measured after a 24h incubation period at 28^0C. The antibiotic susceptibility was determined according to the manufacturer's guidelines.

Results

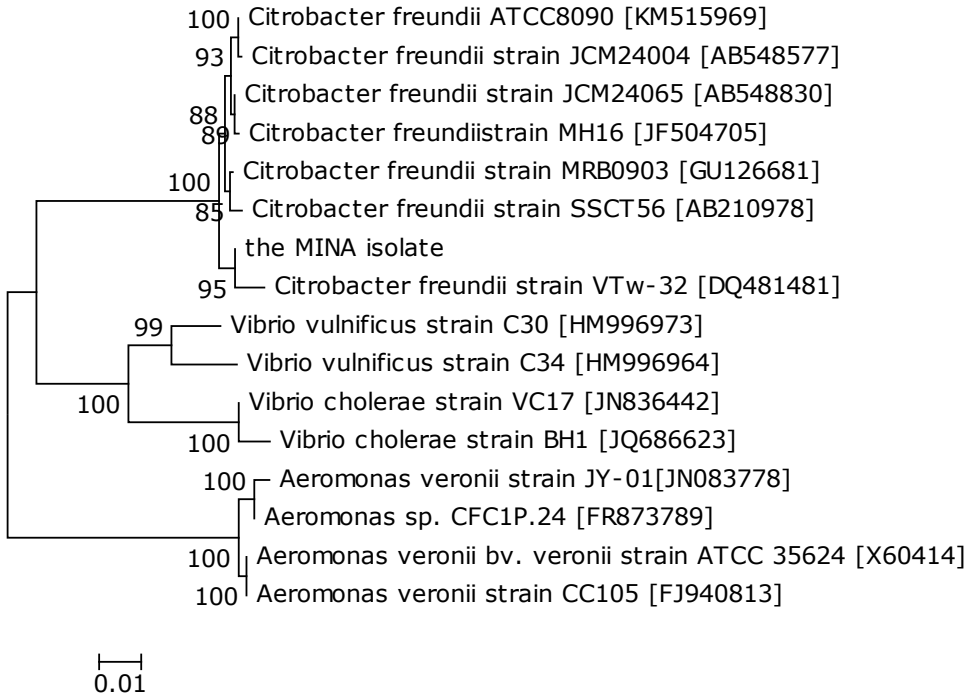
3.1 Identification of the pathogenic isolate. Dominant isolate MINA was isolated from diseased freshwater farmed eels and identified by molecular and phenotypic methods as *C. freundii.* Its near complete 16S rRNA gene sequence (1400 nucleotides) was submitted to GenBank database with accession no. KU249219. 99% similarity was observed in the 16S rRNA gene sequence between the MINA isolate and other *C. freundii* isolates from the GenBank database. The phylogenetic tree confirms that isolate MINA is identified with *C. freundii* strain (Figure 1) and also confirmed by phenotypic features as *C. freundii* (Table 1) with 95% identity compared to the reference strain.

Table 1. Phenotypic characterization of isolate MINA.

Tests	*Reaction*	
	MINA	*C. freundii*[a]
Arginine dihydrolase	R^-	R^-
Cytochrome oxidase	R^-	R^-
β-Galactosidase	R^-	R^+
Gelatinase	R^-	R^-
Lysine decarboxylase	R^-	R^-
Ornithine decarboxylase	R^-	R^-
Tryptophan deaminase	R^-	R^-
Urease	R^-	R^-
Citrate utilization	R^+	R^+
Acetoin production	R^-	R^-
Indole production	R^-	R^-
H_2S production	R^+	R^+
Arabinose fermentation	R^+	R^+
Amygdalin fermentation	R^-	R^-
Glucose fermentation	R^+	R^+
Inositol fermentation	R^-	R^-
Mannitol fermentation	R^+	R^+
Melibiose fermentation	R^+	R^+
Rhamnose fermentation	R^+	R^+
Sucrose fermentation	R^+	R^+
Sorbitol fermentation	R^+	R^+

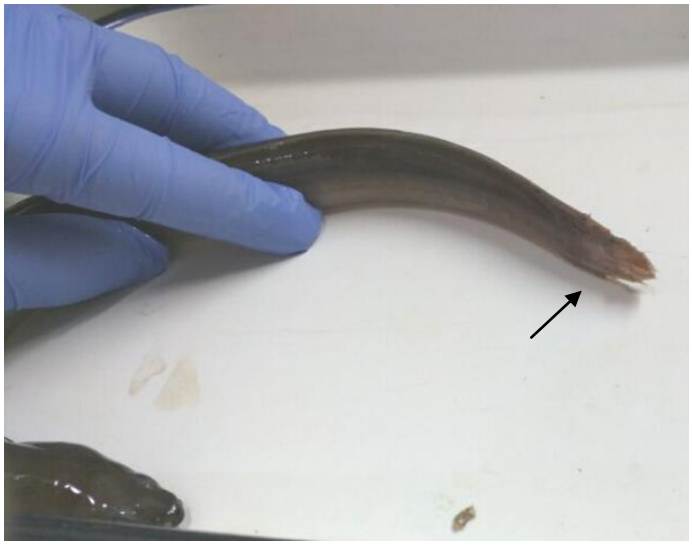
R^+: positive reaction; R^-: negative reaction.[a]The reference strain's data are in accordance with those previously reported (Dong and Cai, 2001).

Figure 1. A 16S rRNA gene tree of 15 known bacteria and the MINA isolate constructed using the neighbour-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.



By reinoculation of the isolated MINA strain it was found to be pathogenic. Morbidity of the eels increased gradually after the inoculation. 35%-100% of the eels inoculated with isolate MINA died at a LD_{50} value of 5.62×10^5 CFU/mL and exhibited symptoms similar to those seen in the originally diseased eels (Figure 2), characterized by rot of the tail fin and muscle (Lu and Han 1989). Re-isolated bacteria from experimentally inoculated eels were identified phenotypically and molecularly as isolate MINA. No clinical signs or mortality appeared in the control group.

Figure 2. Pathological symptoms of the freshwater cultured *A. japonica* suffering from tail rot disease. Arrow indicates the rotten tail.



3.2 Antibiotic sensitivity. Antibiotic sensitivity of isolate MINA is shown in Table 2.

Table 2. Susceptibility of isolate MINA to antibiotics.

Antibiotics	*Content (µg/disc)*	*Inhibition zone diameter (mm)*
Amoxicillin*	10	0±0[R]
Azithromycin	15	0±0[R]
Carbenicillin*	100	0±0[R]
Cefaran	30	0±0[R]
Cefobid	75	0±0[R]
Ceftazidime	30	0±0[R]
Chloromycetin	30	0±0[R]
Ciprofloxacin	5	13.81±0.44[R]
Clindamycin	2	0±0[R]
Cotrimoxazole*	23.75/1.25	0±0[R]
Doxycycline*	30	0±0[R]
Enrofloxacin*	5	9.79±0.38[R]
Erythromycin	15	0±0[R]
Furantoin	30	17.59±0.47[S]
Gentamicin*	10	21.50±0.71[S]
Lincomycin	2	0±0[R]
Medemycin	30	0±0[R]
Neomycin*	30	19.18±1.11[S]
Netilmicin	30	21.00±0[S]
Norfloxacin	10	8.22±1.05[R]
Ofloxacin	5	11.80±0.30[R]
Polymyxin B*	30	13.87±0.51[S]
Rifampicin	5	0±0[R]
Spectinomycin	100	16.25±1.06[I]
Sulfamethoxydiazine*	5	0±0[R]
Vancomycin	30	0±0[R]

Data are presented as the mean ± standard deviation; [S]Sensitive; [I]Intermediately sensitive; [R]Resistant.*Antibiotics for aquaculture use.

The data indicates that isolate MINA is sensitive to furantoin, gentamicin, neomycin, netilmicin, polymyxin B, intermediately sensitive to spectinomycin, and resistant to another twenty tested antibiotics suggesting that isolate MINA has developed resistance to penicillin, quinolone and sulfonamide antibiotics for aquacultural use, as well as to amide alcohol, cephalosporin, glycopeptide and macrolide drugs for veterinary use.

Discussion

In aquaculture, *C. freundii* has been associated with high levels of mortality reported in *Oncorhynchus mykiss* Walbaum (Sanz, 1991; Jeremić et al., 2003), *Oreochiromis niloticus* (L.) (Karunasagar and Pai, 1992), *Cherax quadricarinatus* (Shen et al., 2005), *Rana catesbeiana* (Pasteris et al., 2006), *Eriocheir sinensis* (Chen et al., 2006), *Ctenopharyngodon idellus* (Lü et al., 2011), *Andrias davidianus* (Gao et al., 2012), *Acipenser schrenckii* Brandt (Yang et al., 2013) and *Oreochromis mossambicus* (Thanigaivel et al., 2015). However, there is limited information on *C. freundii* isolates as causal agents for tail rot disease in freshwater cultured eels. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *C. freundii* MINA.

Citrobacter is a genus containing highly virulent species which have the ability to survive serum bactericidal activity, resistance to intracellular destruction by polymorphonuclear leucocytes, and cell surface hydrophobicity (Nayar et al., 2013). Diseases caused by *C. freundii* are usually associated with the production of these virulent factors. In the present study, the MINA isolate was found to cause mortality in healthy *A. japonica* with a LD_{50} value of 5.62×10^5 CFU/mL demonstrating the potential threat of *C. freundii* to freshwater farming of *A. japonica*. Apart from the virulence of the

MINA isolate, other secondary factors such as use of contaminated feed and inferior farming water quality may induce tail rot disease in *A. japonica*; these factors are causes of concern.

Antibiotic resistance in *C. freundii* has been reported in aquaculture as a result of the widespread use of antibiotics. A *C. freundii* isolate from diseased *Acipenser schrenckii* Brandt has been found to be resistant to amoxicillin, carbenicillin, and doxycycline (Yang et al., 2013). A *C. freundii* isolate from diseased *Pelodiscus sinensis* increased resistance to carbenicillin and gentamicin (Mao et al., 2015). We found the MINA isolate in our study to be resistant to multiple antibiotics including amoxicillin, carbenicillin, cotrimoxazole, doxycycline and sulfamethoxydiazine used in fish farming regions, suggesting that overuse of antibiotics may have contributed to the outbreak of this disease.

In conclusion, the present study has shown *C. freundii* isolate as a causal agent for tail rot disease in freshwater cultured *A. japonica*. The pathogenicity and multiple drug resistance of the MINA isolate support the evidence that this infection is an increasing threat to eel farming.

Acknowledgments

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