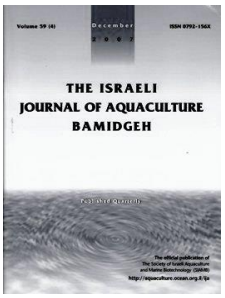

Shewanella putrefaciens: an emerging pathogen for farmed yellow catfish *Pelteobagrus fulvidraco*

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Abstract

Bacteriosis has caused significant economic losses in farmed yellow catfish *Pelteobagrus fulvidraco*. Information is limited on *Shewanella putrefaciens* as a pathogen for yellow catfish. In this study, a virulent strain, temporarily named Y2, was isolated from diseased yellow catfish, identified phenotypically and molecularly as *S. putrefaciens*. A phylogenetic tree was constructed to examine isolate Y2 and compare it to other known isolates. In addition, isolate Y2 is apparently susceptible to aminoglycosides and tetracycline drugs for veterinary use in aquaculture as revealed when screened against a range of common antibiotics. To the best of our knowledge, this is the first report of *S. putrefaciens* as an emerging pathogen for cultured yellow catfish.

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Introduction

Yellow catfish *Pelteobagrus fulvidraco* is an important economic freshwater fish species widely cultivated in China, Japan, Korea, and Southeast Asian countries (Li, 2000). In China, the yellow catfish industry has grown rapidly and is highly profitable (Yang et al., 2009). Production increased to over 355,000 tons in 2015 (Ministry of Agriculture of China, 2016). When cultured intensively however, this industry has been seriously affected by bacterial diseases (Ye, 2009). Bacteriosis should be given more attention to enable further sustainable development of this industry. Studies have shown that several bacterial pathogens such as *Aeromonas hydrophila* (Liu et al., 2015), *Edwardsiella tarda* (Deng et al., 2008), *Edwardsiella ictaluri* (Ye, 2009) and *Vibrio mimicus* (Zhang et al., 2014) can cause infections in yellow catfish. However, there are few reports on *Shewanella putrefaciens* infection in farmed yellow catfish.

In June 2017, a severe disease characterized by hemorrhagic skin spots appeared in yellow catfish in major culture regions of Zhejiang and Fujian provinces, China. The disease is highly infectious and lethal, causing over 80% mortality. In the present study, we isolated and identified a *S. putrefaciens* pathogen as a causative agent for this disease, and determined its taxonomy and antibiotic susceptibility. As far as we know, this is the first report of *S. putrefaciens* as a pathogen for yellow catfish.

Materials and methods

Fish samples. Twenty diseased yellow catfish averaging 43.1±2.8 g were sampled from infected ponds of a fish farm in Huzhou, Zhejiang China in June 2017. The farm had forty acres of ponds with yellow catfish stocked at an initial rearing density of 5,000 juveniles per acre. Water quality during the disease outbreak was pH 8.51, 0.30 mg/L total ammonia, 0.04 mg/L nitrite, and 5.56 mg/L dissolved oxygen. Diseased samples were placed in sterile bags, kept in ice, and transported to the laboratory.

Bacterial isolation. Each sampled diseased yellow catfish was externally disinfected with 75% alcohol, and dissected. Samples from livers, kidneys, and spleens, of diseased yellow catfish were streaked onto nutrient brain heart infusion agar (BHIA) plates (Sinopharm Chemical Reagent Co., Ltd.) before conducting careful detection of parasites and viruses using traditional methods as described by Huang et al. (2010) and Ma et al. (2015). After incubation for 24h at 28^0C, the dominant uniform isolates were purified by streaking and re-streaking onto BHIA plates. Pure isolates of the predominant uniform colonies were stored at -80^0C supplemented with 15% glycerol. A representative of the dominant isolates, temporarily named Y2, was characterized further in the present study.

Bacterial identification: Molecular identification. The extraction of genomic DNA from isolate Y2, as well as PCR amplification and sequencing of its 16S rRNA gene were performed as described by Qin et al. (2012). The near complete 16S rRNA gene sequence was assembled using Editseq and Seqman in DNAstar 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 neighbour-joining method.

Phenotypic identification. Isolate Y2 was identified phenotypically using API 32E test strips as recommended by Cheng et al. (2013). The test strip was incubated at 37^0C and observed after 24h against the API identification index. The type strain ATCC BAA-1097 of *S. putrefaciens* was used as the control.

Virulence assay. Bacterial virulence was examined by experimentally infecting healthy cultured yellow catfish. One hundred and fifty healthy fish averaging 100±10 g were obtained from Baishazhou fishery Co., Ltd. in Wuhan, China. Their health status was evaluated according to the guidelines recommended by Zheng et al. (2012). The experimental fish were acclimated in ten replicate aquaria (each stocked with fifteen fish) supplied with 50 L of aerated filtered farming water at 25^0C for 14 days. Prior to the bacterial virulence assay isolate Y2 was inoculated onto NA plate, incubated at 28^0C for 24h, and washed with normal saline into 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 fifteen healthy fish were innoculated by intraperitoneal injection with 0.2 mL of isolate Y2 at cell densities of 4.0 $\times 10^4$ CFU/mL to 4.0 $\times 10^7$ CFU/mL. Another two replicates of fifteen healthy fish exposed to the same experimental conditions and injected intraperitoneally with 0.2 mL of normal saline served as the control. Experimental fish were kept at 25^0C and observed daily for seven days without feeding and water change. Any dead fish were immediately removed and sampled to confirm if mortality was caused specifically by the inoculated isolate. The mean lethal dose (LD_{50}) value is calculated according to the Graphical Probit Method as recommended by Ogbuagu & Iwuchukwu (2014).

Antibiotic sensitivity assay. The antibiotic sensitivity of isolate Y2 was assayed on NA plates using the Kirby-Bauer disk diffusion method as described by Joseph et al. (2011). Thirteen antibiotic discs were acquired from Hangzhou Tianhe Microorganism Reagent Co., Ltd. The inhibition zones were measured after a 24h incubation period at 28^0C. The antibiotic susceptibility was determined according to the manufacturer's guidelines.

Results

Bacterial identification. A dominant isolate Y2 was isolated from the diseased yellow catfish and identified by molecular and phenotypic methods as *S. putrefaciens.* Its near complete 16S rRNA gene sequence (1400 nucleotides) was submitted to GenBank database with the accession no. MF401513. A similarity of 99% was observed in the 16S rRNA gene sequence between the Y2 isolate and other *S. putrefaciens* isolates from the GenBank database. The phylogenetic tree confirms that the isolate Y2 is identified with *S. putrefaciens* strain (Figure 1).

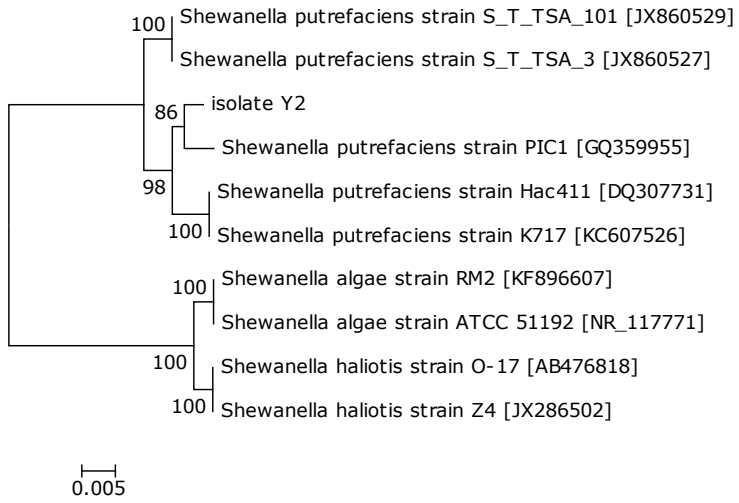

Figure 1. A 16S rRNA gene tree of 9 known bacteria and the Y2 isolate constructed using the neighbour-joining method. The bootstrap values (%) are shown besides the clades; accession numbers are indicated beside the names of strains, and scale bars represent distance values.

This was confirmed by the phenotypic features as *S. putrefaciens* (Table 1) with 96.88% identity compared to the reference strain. No parasites and viruses were detected in the diseased yellow catfish from which the isolate Y2 was obtained.

Table 1. Phenotypic characteristics of isolate Y2.

Tests	*Reaction*	
	Y2	*ATCC BAA-1097*
Arginine dihydrolase	R^-	R^-
Lysine decarboxylase	R^-	R^-
Lipase	R^-	R^-
L-aspartate aminase	R^+	R^+
N-acetyl-β-glucosaminidase	R^+	R^+
α-galactosidase	R^-	R^-
α-glucosaccharase	R^-	R^-
α-maltosidase	R^-	R^-
β-galactosidase	R^-	R^-
β-glucosaccharase	R^-	R^-
β-glucuronidase	R^-	R^-
Urease	R^-	R^-
Orinithine decarboxylase	R^+	R^-
Indole production	R^-	R^-
Malonate utilization	R^+	R^+
Acid production from		
Adonitol	R^-	R^-
Galacturonic acid	R^-	R^-
Inositol	R^-	R^-
L-arabinose	R^-	R^-
L-arabitol	R^-	R^-
L-rhamnose	R^-	R^-
D-arabitol	R^-	R^-
D-cellobiose	R^-	R^-
D-glucose	R^-	R^-
D-maltose	R^-	R^-
D-mannitol	R^-	R^-
D-sorbitol	R^-	R^-
D-sucrose	R^-	R^-
D-trehalose	R^-	R^-
5-ketone-potassium gluconate	R^-	R^-
Palatinose	R^-	R^-
Sodium pyruvate	R^-	R^-

R^+: positive reaction; R^-: negative reaction.

Isolate Y2 was virulent to yellow catfish with a LD_{50} value of 7.56×10^5 CFU/L (Table 2). The infected fish exhibited the similar clinical symptom to that seen in the originally diseased fish (Figure 2). When fish were challenged with a concentration of 4.0×10^7 CFU/mL, acute mortality was observed. Also isolate Y2 could be re-isolated from experimentally dead fish. No clinical signs or mortality were noted in the control fish.

Table 2. Cumulative mortality of experimental yellow catfish infected by the isolate Y2.

Group	*Concentration (CFU/mL)*	*Fish no.*	*Dead fish no. on day after challenge*							*Average cumulative mortality (%)*	*LD_{50} value (CFU/mL)*
			1	*2*	*3*	*4*	*5*	*6*	*7*		
Control	0	15	0	0	0	0	0	0	0	0	7.56×10^5
		15	0	0	0	0	0	0	0		
Treatment 1	4.0×10^4	15	0	1	1	1	0	0	0	20	
		15	0	0	1	1	1	0	0		
Treatment 2	4.0×10^5	15	0	2	1	1	1	1	0	40	
		15	1	1	2	0	2	0	0		
Treatment 3	4.0×10^6	15	3	4	2	1	1	0	0	70	
		15	2	3	2	1	1	1	0		
Treatment 4	4.0×10^7	15	4	7	2	2	0	0	0	100	
		15	5	8	1	1	0	0	0		

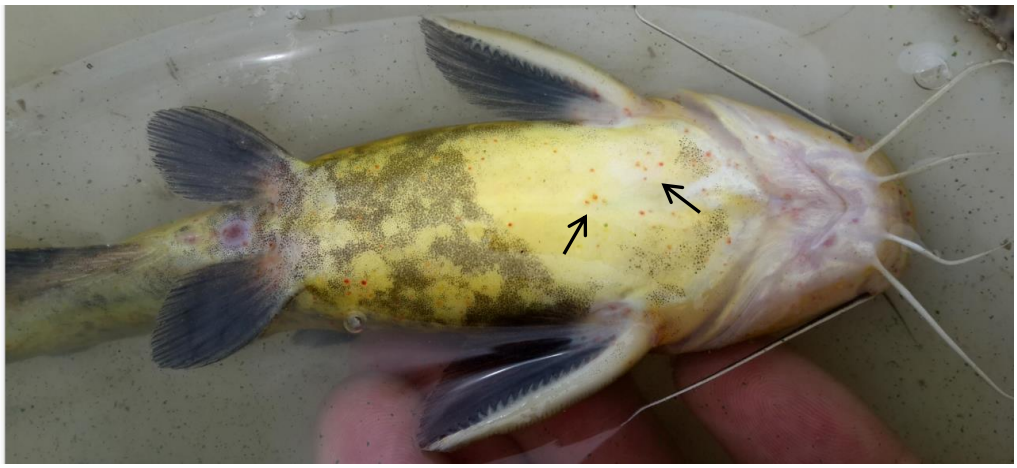

Fig. 2. Pathological symptoms of diseased yellow catfish: arrows show hemorrhagic spots on skin.

Antibiotic susceptibility. The antibiotic sensitivity of isolate Y2 is shown in Table 3. The data indicate that isolate Y2 is sensitive to amikacin, cefradine, cefotaxime, doxycycline, furazolidone, gentamycin, neomycin, tobramycin, intermediately sensitive to florfenicol, and resistant to the penicillins, sulfonamides antibiotics. This suggests that isolate Y2 has not developed resistance to aminoglycosides and tetracycline antimicrobials.

Table 3. Susceptibility of isolate Y2 to antibiotics.

Antibiotics	*Content (μg/disc)*	*Inhibition zone diameter (mm)*
Amikacin	30	24.83 ± 0.63^{S}
Amoxicillin	20	0 ± 0^{R}
Cefradine	30	27.13 ± 0.19^{S}
Cefotaxime	30	34.60 ± 0.41^{S}
Doxycycline*	30	23.95 ± 0.23^{S}
Florfenicol*	75	15.60 ± 0.22^{I}
Furazolidone	300	22.85 ± 0.19^{S}
Gentamycin	10	16.07 ± 0.14^{S}
Neomycin*	30	27.71 ± 0.47^{S}
Oxacillin	1	0 ± 0^{R}
Penicillin	10IU	9.27 ± 0.11^{R}
Sulfamethoxazole*	300	9.64 ± 0.52^{R}
Tobramycin	10	15.87 ± 0.59^{S}

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

Discussion

S. putrefaciens as a pathogen in aquaculture has been documented as causing mortality in *Carassius auratus gibelio* (Altun et al., 2013; Qin et al., 2012), *Babylonia arelolata* (Li et al., 2013), *Oreochromis niloticus* (El-Barbary, 2017), *Anguilla anguill*a L. (Esteve et al., 2017), *Cyprinus carpio* L. and *Oncorhynchus mykiss* (Kozińska & Pekala, 2004; Pekala et al., 2015). However, there is limited information on *S. putrefaciens* as a causal agent for cultured yellow catfish. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *S. putrefaciens* Y2. To our knowledge, this is the first report of a *S. putrefaciens* pathogen as a causative agent for farmed yellow catfish.

Various factors are involved in the pathogenesis of fish shewanellosis, including β-hemolysin production that is closely related to the pathogenicity in *S. putrefaciens* (El-Barbary, 2017) and external stressors that result in reduced immunity in fish (Kozińska & Pekala, 2004). Diseases caused by *S. putrefaciens* are usually associated with these factors. In the present study, the Y2 isolate of *S. putrefaciens* attained an LD_{50} mortality in healthy yellow catfish when challenged with a concentration of 7.56×10^{5} CFU/mL. This

further demonstrates the potential threat of Y2 to yellow catfish farming. Apart from the virulence of the Y2 isolate, there might be other secondary factors that induce shewanellosis in yellow catfish, such as high density stocking (Qin et al., 2012) and hypoxic conditions (Esteve et al., 2017); these should also be raised as concerns.

Intensive fish farming has resulted in the massive use of antimicrobials for treatment of fish bacteriosis (Hu et al., 2015). In our study, the Y2 isolate showed resistance to sulfamethoxazole. The same susceptibility has also been observed in *S. putrefaciens* isolated from *Carassius auratus gibelio* (Qin et al., 2012), *Cyprinus carpio* L. and *Oncorhynchus mykiss* (Kozińska & Pekala, 2004). The Y2 isolate exhibited sensitivity to doxycycline and neomycin used in the fish farming regions, suggesting that the outbreak of this disease may not have resulted from abuse of these antibiotics.

In conclusion, the present study for the first time reports a *S. putrefaciens* isolate as a causal agent for cultured yellow catfish. The pathogenicity of the Y2 isolate supports this infection as an emerging threat in yellow catfish farming.

Acknowledgments

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