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Shewanella algae: an emerging causative agent for ulcer disease in freshwater-farmed American eel *Anguilla rostrata*

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Key words: Ulcer disease; *Anguilla rostrata*; *Shewanella algae*; Antibiotic susceptibility.

Abstract

Ulcer disease has resulted in substantial economic losses in eel aquaculture. Yet only scarce information is available on *Shewanella algae* as a possible causal agent for this disease. In this study, a virulent strain, temporarily named MY, was isolated from diseased American eel, *Anguilla rostrata,* suffering from ulcer disease and was identified as *S. algae* through molecular and phenotypic methods. A phylogenetic tree was constructed to examine MY and compare it to other known isolates. Besides, isolate MY has exhibited susceptibility to aminoglycosides and sulfonamides drugs for veterinary uses in aquaculture, as revealed when screened against a range of conventional antibiotics. To the best of our knowledge, this is the first report of ulcer disease caused by *S. algae* in American eel.

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Introduction

American eel *Anguilla rostrata* is widely cultivated in many countries such as America, Canada, China, Greenland and Guyana (Chen et al., 2005; Kwak et al., 2019). Especially in China, with the rapid development of farming techniques, *A. rostrata* has been cultured successfully in freshwater (Lin et al., 2006) and has become one of the most important commercial fish species which has brought a high profit in recent years (Xu et al., 2012). Its production has increased to over 89,000 tons in 2017 (Ministry of Agriculture and Rural Affairs of China, 2018; Ye et al., 2019). However, under intensive culture, this industry has been severely affected by bacterial diseases (Guo et al., 2010; Xu et al., 2017). Thus, more attention should be paid to bacteriosis to enable further development of this industry.

Ulcer disease is one of the most important infectious bacterial diseases in eel aquaculture (Yang et al., 2015). So far, several bacterial pathogens such as *Aeromonas hydrophila*, *Edwardsiella tarda,* and *Vibrio vulnificus* have been reported to cause this disease (Zheng et al., 1999; Zheng et al., 2005; Lei et al., 2012; Xu et al., 2017). *Shewanella algae* is a Gram-negative, rod-shaped, motile bacterium, which is widely distributed in freshwater and marine environments (Srinivas et al., 2015; Torri et al., 2018; Tseng et al., 2018), and it has emerged as an aquaculture pathogen, which differs from *Shewanella putrefaciens* in its ability to produce β-haemolysis (Khashe et al., 1998; Holt et al., 2005; Lee et al., 2019). The occurrence of *S. algae* infections is most commonly found in warm waters with a temperature of 30^0 C, pH 7 and salinity of 30 ppt (Holt et al., 2005; Han et al., 2017; Tseng et al., 2018). However, little information is available on *Shewanella algae* as a causal agent for ulcer disease in American eel.

This study aims to characterize the phenotype, taxonomic position and antibiotic susceptibility of this strain. As far as we know, this is the first report of *S. algae* as a pathogen of ulcer disease in American eel.

Materials and methods

American eel samples

Twenty-one-year-old diseased freshwater-cultured American eel averaging 100.3±1.5 g suffering from ulcer disease were sampled from an eel farm in Fujian China during May 2018. The farm had 1000 square meters of ponds with American eel stocked at an initial rearing density of 550 juveniles per square meter. The water quality during the disease outbreak was 7.57 pH, 0.08 mg L-1 total ammonia, 0.05 mg L-1 nitrite and 7.21 mg L-1 dissolved oxygen. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory, according to Cao et al. (2016).

Isolation of Bacteria

Each sampled diseased eel was externally disinfected with 75% alcohol and dissected, according to Yang et al. (2017). Before conducting a careful examination of parasites and viruses using traditional methods as described by Huang et al. (2010) and Deng et al. (2009), a section of 0.1 g of ulcerative muscle and 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, as recommended by Ma et al. (2018). A representative of the predominant isolates, temporarily named MY, was characterized further in the present study.

Identification of the pathogen

Molecular identification

The extraction of genomic DNA from the MY isolate, as well as PCR amplification and sequencing of its 16S rRNA gene, were performed according to Li et al. (2015). The nearly 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 nearly complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbor-joining method.

Phenotypic identification

The isolate MY was identified phenotypically by API 20E system recommended by Santos et al. (1993), where the isolate MY was grown on NA plates at 280C for 24h, and the bacterial suspension was then used to inoculate the Analytical Profile Index (API 20E) test strips (Biomerieux, France) following the manufacturer's instruction. The plate was incubated at 37^0 C and observed after 18h for checking against the API identification index and database. Information related to *S. algae* previously reported (Li et al., 2015; Cao et al., 2018) serves as a reference.

Bacterial virulence assay

The experiment was conducted in strict accordance with the Regulations on Experimental Animals Administration of China (Publication No. 676). It was approved by the Institutional Animal Ethics Committee of Shanghai Ocean University with permission No. SHOU-DW-2019-034. Bacterial virulence was examined by experimentally infecting healthy freshwater cultured American eel. One hundred healthy American eel averaging 70.3±1.6 g were obtained from an eel farm in Guangdong China. The experimental fish were acclimated in ten replicate aquaria (ten fish per aquarium) supplied with 100 L of aerated filtered farming water at 28^0 C for 14 days. Before the bacterial virulence assay, the isolate MY was inoculated onto the NA plate, incubated at 28^0 C for 24 h, then washed with normal saline into a sterile tube. Its cell density was determined by counting

colony-forming units after a ten-fold serial dilution in sterile distilled water, as described by Han et al. (2018). Two replicates of ten healthy fish were challenged by intramuscular injection (Chen et al., 2015) with 0.1 mL of the isolate MY at a concentration of 2.0 ×104 CFU mL-1 to 2.0 ×107 CFU mL-1. Another two replicates of ten healthy fish exposed to the same experimental conditions and injected intramuscularly 0.1 mL of normal saline remained unchallenged and served as control. The experimental fish were kept at 280C and observed daily for seven days without feeding and water change. Any dead fish were immediately removed and sampled to re-isolate and confirm if the challenge isolate explicitly caused the mortality, according to Han et al. (2017). Briefly, the livers of the dead fish were sampled on NA plates to re-isolate the challenge isolate, which was identified phenotypically and molecularly, as described above. The mean lethal dose (LD50) value is calculated using the linear regression method, as recommended by Spielmann et al. (1999).

Antibiotic sensitivity assay

The antibiotic sensitivity of isolate MY was assayed on NA plates using the Kirby-Bauer disk diffusion method, as recommended by Cen et al. (2019). Twenty-one antibiotic discs were acquired from Hangzhou Binhe Microorganism Reagent Co., Ltd. The zones of inhibition were measured after a 24h incubation period at 28^0 C. The antibiotic susceptibility was determined according to the manufacturer's guidelines.

Results

Identification of the pathogenic isolate

Only a pathogenic isolate, temporarily named MY, was isolated from diseased freshwater-farmed American eel and identified by molecular and phenotypic methods as *S. algae*. Its near-complete 16S rRNA gene sequence (1400 nucleotides) was submitted to the GenBank database with the accession no. MN795538. The similarity of 99% to 100% is observed in the 16S rRNA gene sequence between the MY isolate and other *S. algae* isolates from the GenBank database. The phylogenetic tree confirms that isolate MY is identified with *S. algae* strain (Figure 1). Also, isolate MY was confirmed by the phenotypic features as *S. algae* (Table 1) with 100% identity compared to the reference strain. No parasites and viruses were detected in the diseased American eel from which isolate MY was isolated.

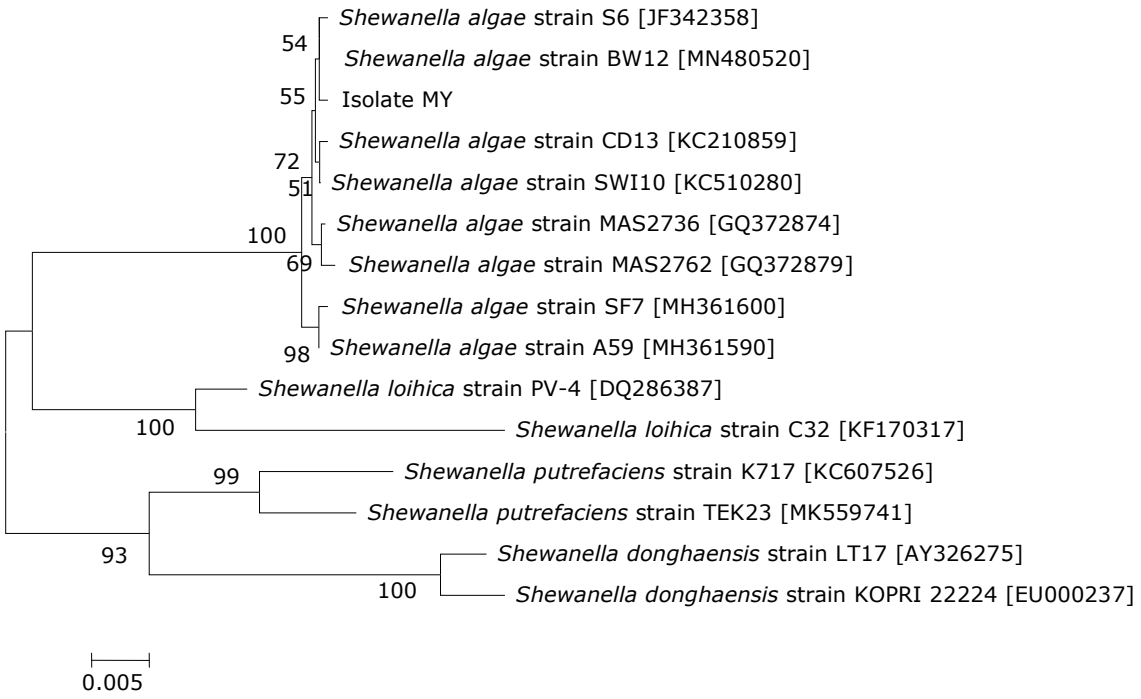

Fig. 1. A 16S rRNA gene tree of 14 known bacteria and the MY isolate constructed using the neighbor-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

Table 1. Phenotypic characterization of isolate MY.

Tests	Reaction	
	Isolate MY	*S. algae*[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 (Li et al., 2015; Cao et al., 2018).

Isolate MY is found to be pathogenic in an experimental challenge. The death of the experimental fish was increased gradually over time after the challenge. 15%-100% of the experimental fish challenged with isolate MY died at an LD50 value of 4.42×105 CFU mL-1 (Table 2) and exhibited rot and necrosis of muscles, similar to that seen in the originally diseased American eel (Figure 2). Also, the re-isolated bacteria from the experimentally dead fish are identified phenotypically and molecularly as isolate MY. No clinical signs or mortality are noted in the control of the American eel.

Table 2. Cumulative mortality of experimental American eel infected by isolate MY.

Group	Concentration (CFU mL^{-1})	Fish no.	Dead fish no. on day after challenge							Average cumulative mortality (%)	LD_{50} value (CFU mL^{-1})
			1	2	3	4	5	6	7		
Control	0	10	0	0	0	0	0	0	0	0	4.42×10^5
		10	0	0	0	0	0	0	0		
Treatment 1	2.0×10^4	10	0	0	1	1	0	0	0	15	
		10	0	0	0	1	0	0	0		
Treatment 2	2.0×10^5	10	0	1	1	1	0	0	0	30	
		10	1	1	0	1	0	0	0		
Treatment 3	2.0×10^6	10	2	2	1	1	1	0	0	75	
		10	2	1	2	2	1	0	0		
Treatment 4	2.0×10^7	10	4	3	2	1	0	0	0	100	
		10	5	3	2	0	0	0	0		

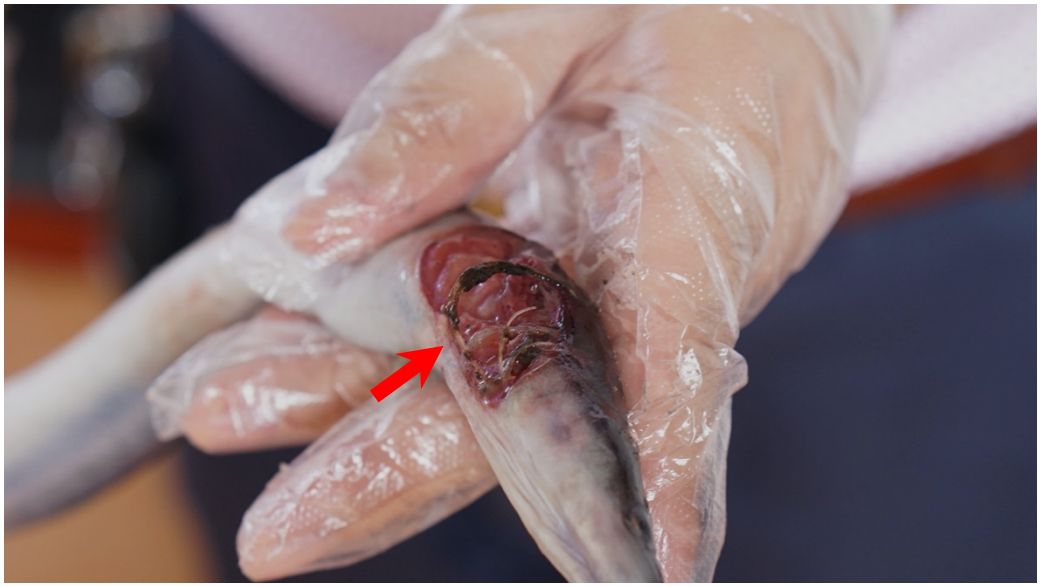

Fig. 2. Pathological symptoms of the freshwater-cultured American eel suffering from ulcer disease. Arrow shows the rotten and necrotic muscle.

Antibiotic sensitivity

The antibiotic sensitivity of isolate MY is shown in Table 3. The data indicate that isolate MY is sensitive to ciprofloxacin, cotrimoxazole, enrofloxacin, erythromycin, florfenicol, furazolidone, gentamycin, neomycin, netilmicin, norfloxacin, ofloxacin, polymyxin B, streptomycin, sulfamethoxydiazine, and intermediately sensitive to lincomycin, and resistant to ampicillin, azithromycin, ceftazidime, doxycycline, rifampicin, tetracycline. Therefore, we suggest that isolate MY did not develop resistance to aminoglycosides and sulfonamides drugs used in eel aquaculture.

Table 3. Susceptibility of isolate MY to antibiotics.

Antibiotics	Content (μg/disc)	Inhibition zone diameter (mm)
Ampicillin	10	8.05±0.07^{R}
Azithromycin	10	8.75±0.35^{R}
Ceftazidime	30	11±0^{R}
Ciprofloxacin	5	26.1±0.57^{S}
Cotrimoxazole*	23.75/1.25	23.5±1.41^{S}
Doxycycline*	30	11.1±0.57^{R}
Enrofloxacin	5	27.15±0.21^{S}
Erythromycin	15	28.15±0.21^{S}
Florfenicol*	30	35.55±0.64^{S}
Furazolidone	30	19±0^{S}
Gentamycin	10	22.85±0.21^{S}
Lincomycin	2	10.1±0.14^{I}
Neomycin*	30	26.65±0.50^{S}
Netilmicin	30	27.25±0.35^{S}
Norfloxacin	10	22.1±0.14^{S}
Ofloxacin	5	25.75±0.35^{S}
Polymyxin B	30	12±0^{S}
Rifampicin	5	6.5±0.71^{R}
Streptomycin	10	26.5±0.71^{S}
Sulfamethoxydiazine*	5	16.5±0.71^{S}
Tetracycline	30	9.25±0.35^{R}

Data are presented as the mean ± standard deviation.

^{S}Sensitive; ^{I}Intermediately sensitive; ^{R}Resistant.

*Antibiotics for eel aquaculture use (Ministry of Agriculture of China, 2013).

Discussion

The association of *S. algae* in aquaculture has been well documented with massive mortality reported in *Scinenops ocellata* (Chen et al., 2003), *Haliotis diversicolor* (Cai et al., 2006; Huang et al., 2018), Babylonia (Li., 2015), *Cynoglossus semilaevis* (Han et al., 2017), *Penaeus vannamei* (Cao et al., 2018) and *Lates calcarifer* (Erfanmanesh et al., 2019). However, there is limited information on *S. algae* isolates as causal agents for ulcer disease in freshwater-cultured eel. In the present work, an *S. algae* pathogen was isolated from American eel suffering from ulcer disease in Fujian China during May 2018, and its phenotype, taxonomic position, and antibiotic susceptibility were characterized. To our knowledge, this is the first report of an *S. algae* pathogen as a causative agent for ulcer disease in freshwater-farmed American eel.

Ulcer disease is usually induced in fish with bacterial pathogens and their virulence factors such as adhesion ability, cytotoxin secretion, enzymatic activity, hemolytic activity, invasiveness (Sakata et al., 1988; Liu et al., 2005; Zhang et

al., 2010; Wang et al., 2012; Xu et al., 2014). *S. algae* is a well-recognized pathogen with virulence factors, including adhesion ability, cytotoxin secretion, enzymatic activity, and hemolytic activity (Lee et al., 2019; Chelsey et al., 2019). Ulcer disease caused by *S. algae* is probably associated with the production of these virulent factors. In the present study, the MY isolate was found to cause mortality in healthy *A. rostrata* with an LD50 value of 4.42×105 CFU mL-1. This phenomenon further demonstrates the potential threat of *S. algae* to freshwater farming of American eel. Apart from the virulence of the MY isolate, there might be other secondary factors that induce ulcer diseases in the American eel, such as reduced health status, misuse of contaminated feed and lack of adequate water disinfection (Lin et al., 2006); these should also be raised as concerns.

Antibiotic resistance in *S. algae* has been reported in aquaculture as a result of the wide use of antibiotics (Cao et al., 2018; Wu et al., 2019). In our study, the MY isolate developed resistance to doxycycline. The same resistance has also been observed in the pathogenic *S. algae* isolated from diseased *P. vannamei* and *L. calcarifer* (Cao et al., 2018; Erfanmanesh et al., 2019). The MY isolate in our study has exhibited sensitivity to aminoglycosides and sulfonamides drugs used in the eel farming regions, suggesting that the outbreak of this disease may not have resulted from the abuse of antibiotics.

In conclusion, the present study, for the first time, reports *S. algae* isolate as a causal agent for ulcer disease in *A. rostrata.* The pathogenicity of the MY isolate supports this infection as an emerging threat in American eel farming.

Acknowledgments

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