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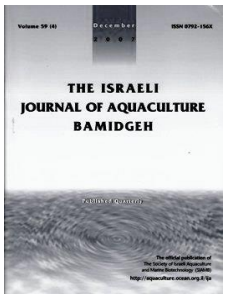

Bacillus mycoides: An Emerging Pathogen of Ulcerative Disease in Farmed Largemouth Bass *Micropterus salmoides*

Haipeng Cao[1,2,3], Yibin Yang[1,4], Liqun Lu[1,2,3], Xianle Yang[1,2,3], Xiaohui Ai[4]*

The first two authors contributed equally to this work.

[1] *National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, Shanghai 201306, P.R. China.*
[2] *Shanghai Collaborative Innovation for Aquatic Animal Genetics and Breeding, Shanghai Ocean University, Shanghai 201306, P.R. China.*
[3] *Shanghai Engineering Research Center of Aquaculture, Shanghai Ocean University, Shanghai 201306, P.R. China.*
[4] *Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan Hubei 430223, P.R. China.*

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Abstract

Ulcerative disease causes significant economic losses in largemouth bass *Micropterus salmoides* production. Yet information reported on *Bacillus mycoides* as a pathogen for largemouth bass is scarce. In this study, a virulent strain, temporarily named LYS1, was isolated from diseased *M. salmoides* suffering from ulcerative disease, identified phenotypically and molecularly as *B. mycoides*. A phylogenetic tree was constructed to examine isolate LYS1 and compare it to other known isolates. In addition, isolate LYS1 appears to be susceptible to aminoglycosides and quinolones drugs for veterinary use in aquaculture as seen when screened against a range of common antibiotics. To the best of our knowledge, this is the first report of *B. mycoides* as a pathogen of ulcerative disease in farmed largemouth bass.

* Corresponding author. Tel: +862781780223; Fax: +862781780223; email: aixh@yfi.ac.cn.

Introduction

Largemouth bass *Micropterus salmoides* is a commercially important freshwater fish widely cultivated in the United States (Heidinger, 2000), China (Lou, 2000), Japan (Maezono & Miyashita, 2003), and many other countries (Welcomme, 1992). In China particularly, the largemouth bass industry has grown rapidly and has been very profitable (Lou, 2000). In 2016, total production reached over 370,000 tons (Ministry of Agriculture of China, 2017). However, in intensive culture, this industry has been seriously affected by bacterial diseases (Xia et al., 2018). Hence, bacteriosis should be given more attention enabling further sustainable development of this industry.

Ulcerative disease is one of the most important infectious bacterial diseases in a wide range of fish including largemouth bass (Saravanan et al., 2013; Xia et al., 2017). This has resulted in significant economic losses in mudfish *Ophiocephalus striatus*, Thai catfish *Clarias batrachus*, crucian carp *Carassius carassius*, goby *Glossogobius giurus* (Llobrera et al., 1987), shing fish *Heteropneustes fossilis* (Bloch)(Rashid et al., 2008), miiuy croaker *Miichthys miiuy* (Hu et al., 2011), Indian catfish *Clarias batrachus* (Linn)(Thomas et al., 2013), and yellow catfish *Pelteobagrus fulvidraco* (Xu et al., 2015). Studies have shown that several bacterial pathogens such as *Aeromonas hydrophila* (Rashid et al., 2008), *Aeromonas veronii* (Xu et al., 2015), *Photobacterium damselae* (Hu et al., 2011) can cause fish ulcerative disease. However, there are few reports on *Bacillus mycoides* as a causal agent for this disease.

In April 2017, an ulcerative disease characterized by skin ulcers appeared in largemouth bass in major culture regions of Guangdong province, China. This disease was highly infectious and lethal, causing over 60% mortality. In the present study, we isolated and identified a *B. mycoides* pathogen as a causative agent for this disease and determined its taxonomy and antibiotic susceptibility. To our knowledge, this is the first report of *B. mycoides* as a pathogen for farmed largemouth bass.

Materials and Methods

Fish samples

Eighteen skin ulcer-infected largemouth bass averaging 72.3±9.5 g were sampled from infected ponds of a fish farm in Foshan, Guangdong China during April 2017. The farm has twenty acres of ponds with largemouth bass stocked at an initial density of 5,000 juveniles per acre. The water quality during the disease outbreak was pH 8.00, 0.20 mg/L total ammonia, 0.25 mg/L nitrite and 4.02 mg/L dissolved oxygen. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

Bacterial isolation

Each sampled diseased fish was externally disinfected with 75% alcohol and dissected. Before conducting a careful detection on parasites and viruses using traditional methods as described by Huang et al. (2010) and Deng et al. (2009), samples from ulerative muscles, livers, and kidneys of diseased fish were streaked onto nutrient agar (NA) (Sinopharm Chemical Reagent Co., Ltd.) with a flamed loop. After incubation for two days at 28^0C, bacteria isolated from the fish were subcultured on the same media plate to check the purity of the isolate. Pure isolates of the predominant uniform colonies were stored at -80^0C supplemented with 15% glycerol. A representative isolate, temporarily named LYS1, was characterized further in the present study.

Bacterial identification

Molecular identification

The extraction of genomic DNA from isolate LYS1, as well as PCR amplification and sequencing of its 16S rRNA gene, were performed as described by Cho et al. (2010). 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 near-complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbour-joining method.

Phenotypic identification

Isolate LYS1 was identified phenotypically using API 50CHB/E test strips as recommended by Nabti et al. (2013). The test strip was incubated at 37^0C and observed after 24h against the API identification index. Information relating to *B. mycoides* (Logan et al., 1985) served as a reference.

Bacterial virulence assay

Bacterial virulence was examined by experimentally infecting healthy cultured largemouth bass. Healthy largemouth bass averaging 95.3±6.3 g were obtained from Baishazhou fishery Co., Ltd. in Wuhan, China, and their health status was evaluated according to the guidelines recommended by Zheng et al. (2012). Forty experimental largemouth bass were acclimated in four replicate aquaria (each stocked with ten fish) supplied with 60 L of aerated filtered farming water at 25^0C for 14 days. Prior to the bacterial virulence assay isolate LYS1 was inoculated onto NA plate, incubated at 28^0C for 24h, and 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. Two replicates of ten healthy fish were challenged by muscular injection with 0.2 mL of isolate LYS1 at a cell density of 1.3×10^6 CFU/mL. Another two replicates of ten healthy fish exposed to the same experimental conditions and injected intramuscularly with 0.2 mL of normal saline that served as control. The 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 re-isolate and confirm if the mortality was caused specifically by the challenge isolate.

Antibiotic sensitivity assay

The antibiotic sensitivity of isolate LYS1 was assayed on NA plates using the Kirby-Bauer disk diffusion method as described by Joseph et al. (2011). Sixteen 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 pathogenic isolate LYS1 was isolated from the diseased largemouth bass and identified by molecular and phenotypic methods as *B. mycoides.* Its near complete 16S rRNA gene sequence (1400 nucleotides) was submitted to GenBank database with the accession no. MK033595. A similarity of 99% to 100% was observed in the 16S rRNA gene sequence between the LYS1 isolate and other *B. mycoides* isolates from the GenBank database. The phylogenetic tree confirmed that the isolate LYS1 was identified with *B. mycoides* strain (Figure 1). This was again confirmed by the phenotypic features as *B. mycoides* (Table 1) with 100% identity compared to the reference strain. No parasites and viruses were detected in the diseased largemouth bass from which the isolate LYS1 was obtained.

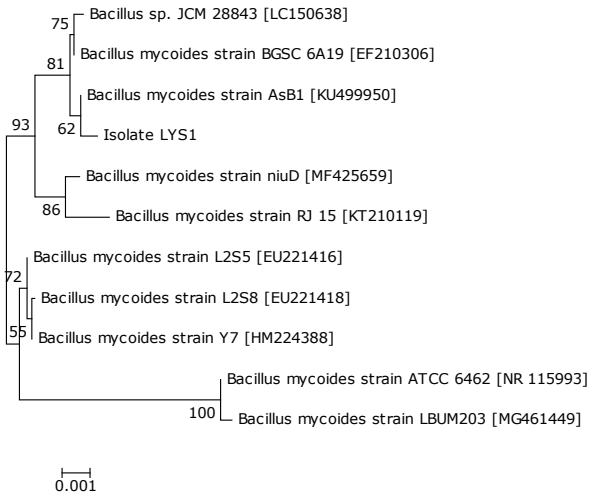

Figure 1. A 16S rRNA gene tree of ten known bacteria and the LYS1 isolate constructed using the neighbour-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

Table 1. Phenotypic characteristics of isolate LYS1.

Tests	*Reaction*	
	LYS1	*B. mycoides*[a]
Amygdaline	R^+	R^+
Amidon	R^+	R^+
Arbutin	R^+	R^+
D-adonitol	R^-	R^-
D-arabinose	R^-	R^-
D-arabitol	R^-	R^-
D-cellobiose	R^-	R^-
D-lactose	R^-	R^-
D-lyxose	R^-	R^-
D-maltose	R^+	R^+
D-mannitol	R^-	R^-
D-mannose	R^-	R^-
D-melezitose	R^-	R^-
D-melibiose	R^-	R^-
D-fructose	R^+	R^+
D-fucose	R^-	R^-
D-glactose	R^+	R^+
D-glucose	R^+	R^+
D-raffinose	R^-	R^-
D-ribose	R^+	R^+
D-sucrose	R^+	R^+
D-sorbitol	R^-	R^-
D-tagatose	R^-	R^-
D-trehalose	R^+	R^+
D-turanose	R^-	R^-
Dulcitol	R^-	R^-
D-xylose	R^-	R^-
Erythritol	R^-	R^-
Esculine	R^+	R^+
Gentiobiose	R^-	R^-
Glycerol	R^+	R^+
Glycogen	R^+	R^+
Inositol	R^-	R^-
Inuline	R^-	R^-
L-arabinose	R^-	R^-
L-arabitol	R^-	R^-
L-fucose	R^-	R^-
L-rhamnose	R^-	R^-
L-sorbose	R^-	R^-
L-xylose	R^-	R^-
Methyl-αD-glucopyranoside	R^-	R^-
Methyl-αD-mannopyranoside	R^-	R^-
Methyl-βD-xylopyranoside	R^-	R^-
N-acethylglucosamine	R^+	R^+
Potassium gluconate	R^-	R^-
Potassium 5-cetogluconate	R^-	R^-
Potassium 2-cetogluconate	R^-	R^-
Salicine	R^+	R^+
Xylitol	R^-	R^-

R^+: positive reaction; R^-: negative reaction.
[a]The reference strain's data are in accordance with those previously reported (Logan et al., 1985)

Isolate LYS1 was found to be pathogenic in an experimental challenge. 85% of the experimental fish challenged with isolate LYS1 died, and the death rate gradually increased over time (Table 2) and also exhibited ulcerative skin lesions**,** similar to those seen in the originally diseased fish (Figure 2). The re-isolated bacteria from experimentally dead fish were identified phenotypically and molecularly as LYS1. No clinical signs or mortality were noted in the control fish.

Table 2. Cumulative mortality of experimental largemouth bass infected by the LYS1 isolate.

Group	*Concentration (CFU/mL)*	*Fish no.*	*Dead fish no. on day after challenge*							*Average mortality (%)*
			1	*2*	*3*	*4*	*5*	*6*	*7*	
Control	0	10	0	0	0	0	0	0	0	0
		10	0	0	0	0	0	0	0	
Treatment	1.3×10^6	10	1	3	4	5	7	7	8	85
		10	2	3	5	6	8	9	9	

Figure 2. Pathological symptoms of the diseased largemouth bass. Arrow shows skin ulcers.

Antibiotic susceptibility

The antibiotic sensitivity of isolate LYS1 is shown in Table 3. The data indicate that the isolate LYS1 is sensitive to ciprofloxacin, enrofloxacin, erythromycin, furazolidone, gentamycin, neomycin, norfloxacin, ofloxacin, streptomycin, and resistant to amoxicillin, ampicillin, ceftazidime, penicillin, sulfamethoxydiazine, tetracycline, trimethoprim-sulfamethoxazole. This suggests that the isolate LYS1 has not developed resistance to aminoglycosides and quinolones antimicrobials used in aquaculture.

Table 3. Susceptibility of isolate LYS1 to antibiotics.

Antibiotics	*Content (μg/disc)*	*Inhibition zone diameter (mm)*
Amoxicillin	10	0 ± 0^R
Ampicillin	10	0 ± 0^R
Ceftazidime	30	0 ± 0^R
Ciprofloxacin	5	26.2 ± 0.3^S
Enrofloxacin*	5	23.1 ± 0.1^S
Erythromycin	15	31.3 ± 0.3^S
Furazolidone	30	20.0 ± 1.3^S
Gentamycin	10	23.9 ± 0.4^S
Neomycin*	30	25.0 ± 0.2^S
Norfloxacin	10	24.5 ± 0.5^S
Ofloxacin	5	28.0 ± 0.1^S
Penicillin	10	0 ± 0^R
Streptomycin	10	23.5 ± 0.5^S
Sulfamethoxydiazine*	5	0 ± 0^R
Tetracycline	30	13.9 ± 0.6^R
Trimethoprim-sulfamethoxazole	23.75/1.25	0 ± 0^R

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

Discussion

The association of *B. mycoides* in aquaculture has been documented with mortality in channel catfish *Ictalurus punctatus* (Goodwin et al., 1994) and soft-shelled turtle *Trionyx sinensis* (Chen et al., 2011). However, there is limited information on *B. mycoides* as a causal agent for cultured largemouth bass. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *B. mycoides* LYS1. To our knowledge, this is the first report of a *B. mycoides* pathogen for farmed largemouth bass.

B. mycoides is a member of the *Bacillus cereus* group with the ability to produce virulence factors including enterotoxins, phospholipases, and endotoxins (Sergeev et al., 2006). Fish diseases caused by *B. mycoides* are usually associated with the production of these virulent factors (Goodwin et al., 1994). In the present study, the LYS1 isolate was found to be pathogenic to healthy largemouth bass with a mortality of 85%. This further demonstrates the potential threat of *B. mycoides* to fish aquaculture. Apart from the virulence of the LYS1 isolate, there might be other secondary factors that induce ulcerative disease in fish such as use of contaminated feed and inferior farming water quality which should also be raised as concerns (Cao et al., 2016).

The development of antimicrobial resistance in bacterial pathogens is a matter of concern. In our study, resistance to penicillins and cephalosporins antibiotics was found in the LYS1 isolate. A similar susceptibility was also reported in *B. mycoides* isolated from environmental sources (Turnbull et al., 2004). In addition, the LYS1 isolate exhibited resistance to sulfonamides drugs used in fish farming regions, suggesting that the outbreak of this disease may have resulted from the abuse of antibiotics.

In conclusion, the present study reports for the first time a *B. mycoides* isolate as a pathogen for cultured largemouth bass. The pathogenicity of the LYS1 isolate supports this infection as an emerging threat in largemouth bass farming.

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