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Control by Herbal Extract of *Serratia marcescens* from Cultured Siberian Sturgeon *Acipenser baerii* Brandt

Haipeng Cao[1,2], Yibin Yang[1], Shan He[1], Weidong Zheng[3], Kai Liu[4], Xianle Yang[1*]

[1] *National Aquatic Pathogen Collection Center, Shanghai Ocean University, Shanghai 201306, China*

[2] *Shanxi Veterinary Drug and Feed Engineering Technology Research Center, Yuncheng 044000, China*

[3] *Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan 430223, China*

[4] *Hangzhou Academy of Agricultural Sciences, Hangzhou 310013, China*

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Abstract

A virulent strain of *Serratia marcescens*, provisionally named SFY, was isolated from farmed Siberian sturgeon *Acipenser baerii* Brandt suffering from mouth-swelling disease and identified using the ATB 32GN system. Its taxonomic position was determined by phylogenetic analysis. A phylogenetic tree constructed using the neighbor-joining method showed that the SFY isolate was the *S. marcescens* strain (GenBank accession no. FJ530951). The strain was resistant to chloramphenicol, erythromycin, furazolidone, sulfamethoxydiazine, tetracycline, and trimethoprim-sulfamethoxazole, and susceptible to ciprofloxacin, enrofloxacin, gentamicin, neomycin, norfloxacin, ofloxacin, and streptomycin. Extracts from 30 Chinese herbs were screened as possible agents for control of the disease. *Fructus mume* extract was the most efficacious agent against the SFY isolate and control bacteria, indicated by its low minimum inhibitory concentration ≤21 mg/ml. At concentrations of 10 and 15 g/kg feed, protective efficacy was 42.68% and 64.64%.

The first two authors contributed equally to this work.
*Corresponding author. Tel: +86-216-1900453, fax: +86-216-1900452, e-mail: xlyang@shou.edu.cn

Introduction

The Siberian sturgeon *Acipenser baerii* Brandt is widely distributed and cultivated in France, Germany, Italy, Spain, and China (Memis et al., 2009). It has become an important commercial fish species especially in China, where over 2,000 tons have been produced since 2000 (Sun et al., 2003). However, bacteriosis has become a major economic problem in Siberian sturgeon culture and bacterial diseases should be given more attention for the sustainable development of the farming industry.

Serratia marcescens is an important fish and human pathogen (Baya et al., 1992; Fedrigo et al., 2011). It is a causative agent in white perch *Morone americanus* Gmelin (Baya et al., 1992) and a potential pathogen in mastitis, bacteremia, urinary tract infections, and wound infections (Bamum et al., 1958; Hejazi and Falkiner, 1997). Diseases caused by *S. marcescens* are attributed to its production of proteases (Baya et al., 1992), multiple antibiotic resistance, and ability to adhere and persist on unanimated surfaces (Hejazi and Falkiner, 1997; Hejazi et al., 2000; Kramer et al., 2006).

Mouth-swelling disease is a serious bacterial disease of sturgeons in China, showing typical symptoms of mouth swelling and hyperemia around the mouth (He and Chen, 2000). In this paper, a virulent *S. marcescens* strain was isolated from diseased Siberian sturgeons with mouth-swelling disease in Quzhou, Zhejiang China. The taxonomic position of the strain was determined by phenotypic and phylogenetic analysis, its antibiotic resistance was examined by the Kirby-Bauer disk diffusion method, and its susceptibility to Chinese herb extracts was assayed to screen potential drugs for the control of mouth-swelling disease.

Materials and Methods

Siberian sturgeon and isolation of bacteria. Thirty-six diseased Siberian sturgeons (11.3±0.2 kg) with typical signs of mouth-swelling disease as described by He and Chen (2000) were sampled and transported to the laboratory from Quzhou Siberian Sturgeon Farm, Zhejiang, China in June 2012 (Fig. 1). Moribund Siberian sturgeons, still ventilating but unable to hold position or remain upright (Hruska et al., 2010), were externally disinfected with 75% alcohol and dissected. Samples (0.2 g) of livers and kidneys were taken to isolate and purify bacteria according to Cao et al. (2010).

Identification of bacteria. The isolates were phenotypically identified using the ATB 32GN system as recommended by Altwegg and Zollinger-Iten (1987). Briefly, the isolate was grown on nutrient agar plates (Sinopharm Chemical Reagent Co., Ltd.) at 28°C for 24 h, then the bacterial suspension was inoculated on an API ID32GN strip (bioMérieux, SA) following the manufacturer's instructions. The strip was incubated at 28°C and observed after 48 h by checking against the API identification index and database. Type strain ATCC8100 of *S. marcescens* was used as the control.

The genomic DNA extract, PCR amplification of the 16S rRNA gene, and sequencing of the isolate were performed according to Cao et al. (2010). The partial 16S rRNA sequence was assembled using MegAlign, Editseq and Seqman software with a Power Macintosh computer. The National Centre for Biotechnology Information (NCBI) database was searched using the Basic Local Alignment Search Tool program. A phylogenetic tree from the partial 16S rRNA sequence of the isolate and its homologous sequences was

Fig. 1. Pathological symptoms of naturally infected Siberian sturgeon. Arrows indicate swollen mouth, hyperemia, and hemorrhage on pectoral fins.

constructed using the neighbor-joining method.

Bacterial virulence assay. After careful examination of the physical appearance, behavior, liver, and kidney for bacterial pathogens as recommended by Zheng et al. (2012), 40 healthy Siberian sturgeons (76±8 g) were obtained from Qiandao Lake Sturgeon Culture Co., Ltd., in Hangzhou, Zhejiang, China. The fish were maintained in four aquaria at 10 fish/aquarium supplied with 100 l dechlorinated tap water at 22°C for 14 days. Prior to the bacterial virulence assay, live cells of the isolate were prepared as described by Cao et al. (2010), and cell density was determined by the dilution and spread plate technique. Two aquaria containing 10 healthy fish, each, were challenged by intramuscular injection with live cells of the isolate at a density of 5.0×10^6 cfu/ml as recommended by Ma et al. (2009). Another two aquaria with 10 healthy fish, each, were intramuscularly injected with the same volume of sterile physiological saline as the control. The injected fish were kept at 22°C and observed daily for seven days. Dead fish were immediately removed for pathogen isolation according to Bucke (1989). Symptoms and mortality were recorded.

Antibiotic resistance assay. Resistance of the isolate to antimicrobial agents was assayed on nutrient agar plates using the Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Thirteen fishery antibiotic discs were obtained from Hangzhou Tianhe Microorganism Reagent Co., Ltd. The diameters of the zones of inhibition against the isolate were measured and recorded after 24-h incubation. Antibiotic resistance was determined following susceptibility criteria of the manufacturer.

Susceptibility to Chinese herb extracts. Thirty Chinese herbs were obtained from Shanghai Fosun Industrial Co., Ltd. Aqueous extracts (1 g/ml) of each herb were prepared in triplicate using the boiling method (Au et al., 2001). The minimum inhibitory concentration (MIC) of each extract against the isolate, i.e., the lowest concentration that prevented visible cell growth, was determined by the dilution plate method (Basile et al., 2006). Three bacterial pathogens (*Aeromonas hydrophila* strain S1, *Aeromonas punctata subsp. caviae* strain XL_2-T, *Plesiomonas shigelloides* strain HK_3-R), previously isolated from septicemia-infected sturgeons and phenotypically and molecularly identified (Cao et al., 2007, 2010), were used as control bacteria.

Protective efficacy assay. Healthy Siberian sturgeons (76±8 g) were obtained from Qiandao Lake Sturgeon Culture Co., Ltd. in Hangzhou, Zhejiang China, and maintained in nine glass aquaria supplied with aerated recycled filtered farm water at 22°C. Each aquarium was randomly stocked with 30 healthy fish. The herb extract (1 g/ml) was manually incorporated into commercial dry pellets to a final concentration of 10 or 15 g/kg feed (low and high doses). Control fish were fed commercial dry pellets without an herbal extract. Each treatment was conducted in triplicate. Fish were fed approximately 2% of their body weight twice a day. After three months, fish in all groups were challenged by intramuscular injection of live cells of the isolate at a cell density of 5.0×10^6 cfu/ml as recommended by Ma et al. (2009), and observed daily for seven days. Dead fish were immediately removed for pathogen isolation as described by Bucke (1989), and mortality was recorded each day for 7 days.

Results

Identification of the isolate. Only one bacterial strain was isolated from the kidney of the diseased fish and it was provisionally named SFY. Of the fish challenged by the SFY isolate, 95% acutely died with the same clinical mouth-swelling disease signs as the original infected fish while no acute mortality or visible changes were observed in the control fish. The ATB 32GN system identified our SFY isolate as an *S. marcescens* strain (Table 1), with 93.8% phenotypic identity to type strain ATCC8100. The partial 16S rRNA sequence (ca. 1.4 kb) of our SFY isolate was submitted to the GenBank database with the accession number KC206270. The similarities between the 16S rRNA sequence of SFY and those of *S. marcescens* strains in the GenBank database were 99-100%. The constructed phylogenetic tree further identified the SFY isolate as GenBank *S. marcescens* strain accession no. FJ530951 (Fig. 2).

Table 1. Phenotypic characterization of our SFY isolate and GenBank strain ATCC8100 of *Serratia marcescens*.

Test item	*Reaction*		*Test item*	*Reaction*	
	SFY	*ATCC 8100*		*SFY*	*ATCC 8100*
D-melibiose	+	-	Malonate	-	-
DL-lactate	+	+	Caprate	+	+
N-acetylglucosamin	+	+	Inositol	+	+
D-glucose	+	+	Acetate	+	+
3-hydroxy-benzoate	-	+	Sucrose	+	+
5-keto-gluconate	+	+	L-fucose	+	+
2-keto-gluconate	+	+	Maltose	+	+
3-hydroxy-butyrate	+	+	L-serine	+	+
4-hydroxy-benzoate	+	+	Mannite	+	+
Rhamnose	-	-	Valerate	-	-
Glycogen	-	-	L-alanine	+	+
D-sorbitol	+	+	Citrate	+	+
Itaconic acid	-	-	D-ribose	+	+
L-arabinose	-	-	Histidine	+	+
Propionate	-	-	Salicin	+	+
Suberate	-	-			

+ = positive, - = negative

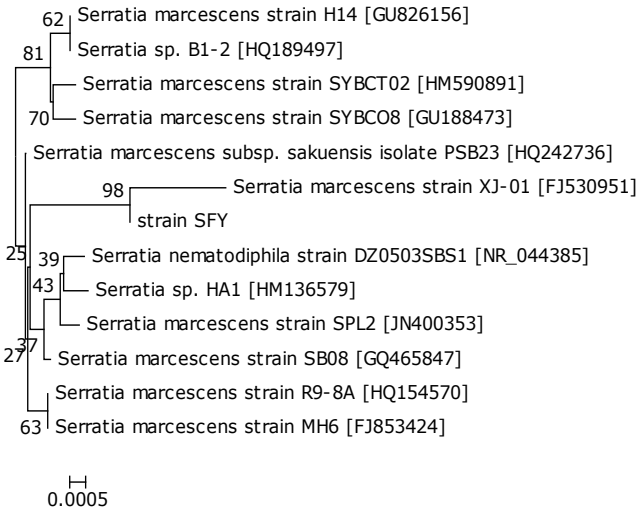

Fig. 2. Constructed 16S rRNA phylogenetic tree of 12 known bacteria and our SFY isolate using the neighbor-joining method. Bootstrap values (%) are shown beside clades, accession numbers are given in brackets, scale bars represent distance values.

Cause of death. As determined by bacteria isolation and molecular identification, the death of all deceased challenged fish was caused by the SFY strain (data not shown).

Antibiotic resistance. The resistance of the SFY isolate to thirteen antimicrobial agents is shown in Table 2.

Susceptibility to herbal extracts. The susceptibility of the SFY isolate to 30 herb extracts is shown in Table 3 (following page). The *Fructus mume* extract had the best potential efficacy, i.e., the inhibition zone with this herbal extract was ≤21 mg/ml, effectively inhibiting the growth of the SFY isolate.

Protective efficacy of Fructus mume. The protective efficacy of the *F. mume* extract against *S. marcescens* infection in Siberian sturgeons is shown in Fig. 3. There was significant protective efficacy against challenge with the SFY strain in fish fed doses of 10 or 15 g/kg feed for three months, i.e., 42.68% and 64.64%, respectively.

Table 2. Susceptibility of the SFY isolate to antimicrobial agents.

Antibiotic agent	*Resistance*	*Content (μg/disc)*	*Inhibition zone (mm)*
Chloramphenicol	R	30	8.75±0.35
Ciprofloxacin	S	5	39.75±1.06
Enrofloxacin	S	5	22.25±0.35
Erythromycin	R	15	0±0
Furazolidone	R	300	9.50±1.41
Gentamicin	S	10	21.75±1.06
Neomycin	S	30	23.75±0.35
Norfloxacin	S	10	33.75±0.35
Ofloxacin	S	5	33.75±0.35
Streptomycin	S	10	21.50±2.12
Sulfamethoxydiazine	R	5	0±0
Tetracycline	R	30	7.75±0.35
Trimethoprim-sulfamethoxazole	R	23.7/1.25	0±0

R = resistant, S = susceptible

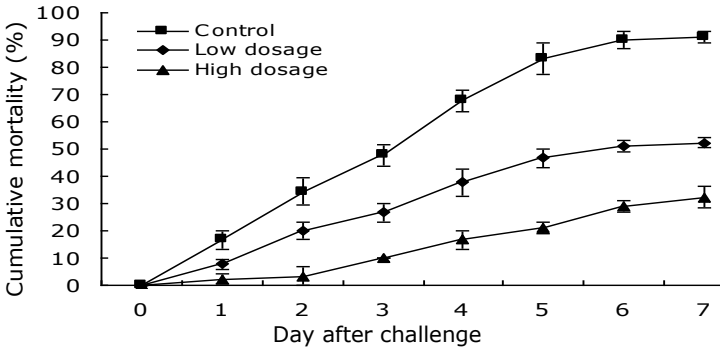

Fig. 3. Protective efficacy of 10 (low dosage) or 15 (high dosage) g *Fructus mume* extract per kg feed on *Serratia marcescens* infection in Siberian sturgeons.

Table 3. Minimum inhibition concentrations (MIC) of Chinese herb extracts against our SFY isolate and three control bacteria.

Chinese herb	*Part used for extract*	*MIC (mg/ml)*			
		Strain SFY	*Strain S1*	*Strain XL_2-T*	*Strain HK_3-R*
Bupleurum chinense	root	1000±0	1000±0	833±289	667±289
Catsia tora Linn	seed	500±0	667±289	500±0	500±0
Cortex cinnamomi	bark	1000±0	667±289	1000±0	1000±0
Eucommia ulmoides	bark	667±289	750±433	667±289	500±0
Exocarpium benincasae	peel	1000±0	583±382	500±0	667±289
Fibraurea tinctoria lour.	stem	1000±0	667±289	833±289	1000±0
Flos carthami	flower	667±289	833±289	667±289	1000±0
Folium artemisiae argyi	leaf	1000±0	667±289	833±289	500±0
Folium isatidis	root	500±0	583±382	500±0	667±289
Folium nelumbinis	leaf	1000±0	1000±0	833±289	667±289
Fructus carpesii	fruit	167±72	275±191	333±144	250±0
Fructus mume	fruit	16±0	21±9	8±0	8±0
Fructus lycii barbari	fruit	125±0	104±36	125±0	167±72
Galla chinensis	fruit	333±144	333±144	250±217	500±0
Herba artemisiae annuae	flower	250±0	333±144	333±144	500±0
Jasminum sambac (L.) *aiton*	flower	167±72	275±191	125±0	250±0
Leguminosae	root	1000±0	833±289	1000±0	500±0
Pericarpium citri reticulatae viride	bark	250±0	333±144	167±72	250±0
Pericarpium granati	bark	667±289	667±289	500±0	833±289
Rhizoma atractylodis macrocephalae	root	250±0	333±144	250±0	250±0
Radix aucklandiae	root	500±0	667±289	333±144	500±0
Radix ophiopogonis	root	63±0	51±18	63±0	63±0
Radix paeoniae rubra	root	500±0	417±144	167±72	125±0
Radix rehmanniae preparata	root	83±36	63±0	63±0	31±0
Radix sanguisorbae	root	667±289	667±289	1000±0	500±0
Radix sophorae flavescentis	root	500±0	500±0	1000±0	1000±0
Radix et rhizoma rhei	root	667±289	500±0	1000±0	833±289
Rhizoma phragmitis	root	1000±0	1000±0	833±289	1000±0
Salvia miltiorrhiza	root	250±0	208±72	250±0	125±0
Trachycarpus fortunei	leaf	250±0	250±0	250±0	333±144

Discussion

Mouth-swelling disease is an important and destructive bacterial disease in farmed sturgeons (He and Chen, 2000). In this study, we isolated a virulent strain of *S. marcescens* from cultured Siberian sturgeons with mouth-swelling disease, and assayed the phenotypic characteristics, taxonomic position, and antibiotic resistance of the strain. Further, an *F. mume* extract was screened as a potential drug for the control of *S. marcescens* infection in farmed Siberian sturgeons.

The excellent performance and accuracy of the ATB 32GN system allows routine identification of gram-negative bacteria in a clinical laboratory (Altwegg and Zollinger-Iten, 1987). Seventy-eight *Serratia* isolates were identified to the species level using the ATB 32GN system (Ulatowska et al., 2000). In the present study, our SFY isolate was identified as a *S. marcescens* strain using the ATB 32GN system and, to better understand the taxonomic position of the SFY isolate, a molecular phylogenetic study was conducted as recommended by Rascoe et al. (2003). Results of the phylogenetic study were consistent with those of the ATB 32GN system and findings of Jin et al. (2010).

The pathogenesis of *S. marcescens* is complex and multi-factorial, involving a number of virulence factors such as serratia proteases and cell-bound hemolysin (Lyerly and Kreger, 1983; Marre et al., 1989; Lin et al., 2010). In the present study, the SFY isolate was virulent to healthy Siberian sturgeons and challenged fish displayed typical symptoms of mouth-swelling disease. There might be other causes or contributing factors to the incidence of mouth-swelling disease, e.g., high fish density, contaminated feed,

low dissolved oxygen concentration, or high ammonia levels (Abulhamd, 2010; Cao et al., 2010). Thus, the pathogenesis of strain SFY needs further exploration.

Antibiotic resistance in *S. marcescens* strains has resulted from the wide use of antibiotics (Sleigh, 1983). Like the SFY isolate in our study, 102 clinical isolates of *S. marcescens* had low resistance to gentamicin (Cooksey et al., 1975). In addition, the SFY isolate was susceptible to other drugs commonly used in the sturgeon fish cultivation region, including enrofloxacin, neomycin, norfloxacin, ofloxacin, and streptomycin. While effective treatments for mouth-swelling disease have been suggested (Qu, 2010), the susceptibility of the SFY isolate to the above drugs indicate that they can be used to prevent and treat outbreaks of mouth-swelling disease in farmed sturgeons.

Herbal extracts can be alternative bacteriosis therapies because of their low toxicity and cost (Direkbusarakom et al., 1998; Muniruzzaman and Chowdhury, 2004; Sharma et al., 2012). *Fructus mume*, the processed unripe fruit of *Prunus mume*, is a medicinal food in Japan and has anti-inflammatory effects in inflammatory bowel disease and macrophage-mediated inflammation (Chen et al., 2011; Jeon et al., 2012). Nine organic acids in its extract have antibacterial activity, i.e., acetic, ascorbic, citric, fumaric, maleic, malic, oxalic, succinic, and tartaric (Gao et al., 2012). The antibacterial mechanisms of these acids are well recognized (Chen et al., 2011) but antibacterial activity drops significantly when the pH rises from 2.943 to 6.965 (Luo and Zhang, 2011). In our study, the *F. mume* extract significantly inhibited growth of the SFY strain and the control bacteria at as low as ≤21 mg/ml, and exhibited significant protective efficacy against experimental infection of Siberian sturgeons, i.e., 42.68% and 64.64% at doses of 10 and 15 g/kg feed, respectively. This may be due to its ability to produce organic acids with antibacterial activities (Chen et al., 2011), and to its immune-enhancing effects on fish (Xiang et al., 2012). Thus, *F. mume* extract is promising as a potential drug for the control of mouth-swelling disease in Siberian sturgeon.

In conclusion, based on phenotypic features and phylogenetic analysis, the causative agent of mouth-swelling disease in Siberian sturgeons is *S. marcescens* and *F. mume* extract is a potential drug for control of the disease.

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