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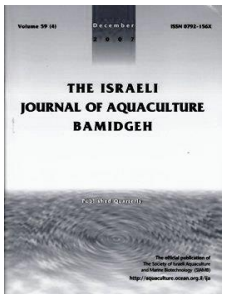

Characterization of *Shewanella sp.* Isolated from Cultured Loach *Misgurnus anguillicaudatus*

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Keywords: *Misgurnus anguillicaudatus*, *Shewanella*, 16S rRNA, gyrB, antimicrobial susceptibility

Abstract

Shewanella infection of fish has become a significant problem in aquaculture. In September 2014, a disease was seen in cultured loach (*Misgurnus anguillicaudatus*) in Xuzhou, central China. A gram-negative bacillus was isolated from the diseased loaches and was tentatively named strain MS1, which was then identified as *Shewanella sp.* by physiological and biochemical characteristics analysis. The strain MS1 showed highest 16S rRNA sequence identities (98.93%, 98.87%) with the latest two species listed (*Shewanella sp.* MR7, *Shewanella sp.* MR4). The phylogenetic tree constructed on the basis of 16S rRNA gene sequences strongly indicated that the strain MS1 is most closely related to the new *Shewanella* strains MR7 and MR4. The isolate MS1 was confirmed as the pathogen of the infected loaches by experimental reinoculation. The strain was susceptible to most antimicrobial agents tested, but resistant to glycopeptides (vancomycin, teicoplanin) and lincosamide (lincomycin, clindamycin). This is the second report on *Shewanella sp.* isolated from the diseased loach.

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Introduction

Cyprinid loach *M. anguillicaudatus* is an economically valuable freshwater fish that is widely distributed throughout China. Bacterial disease outbreaks have caused extensive economic losses in cultured loach (Qin et al., 2008; Qin et al., 2014; Jun et al., 2010). Some bacterial pathogens which have been isolated from the diseased loaches include *Aeromonas, Vibrio* and *Shewanella* (Jun et al., 2010; Bing et al., 2009; Qin et al., 2014). *Shewanella* is a genus of Gram-negative bacilli belonging to the family *Alteromonadaceae* (Bowman, 2005), and *Shewanella spp.* are widespread in aquatic environments (Gentile et al., 2003; Wei and Zhang, 2007; Kim et al., 2011; Li et al., 2014). *Shewanella* was one of the predominant species in the intestinal flora of marine and freshwater organisms, including sardine (*Sardinops melanostictus*), benthonic organism (*Munida subrrugosa*), ayu (*Plecoglossus altivelis*) and rainbow trout (*Oncorhynchus mykiss*) (Lee et al., 2009; Cristóbal et al., 2008; Morohoshi et al., 2005; Spanggaard et al., 2000). Studies have indicated that several *Shewanella* species are associated with fish infections, and *S. putrefaciens* was isolated from European sea bass (*Dicentrarchus labrax*), gibel carp (*Carassius auratus gibelio*) and rainbow trout (*Oncorhynchus mykiss*) (Korun et al., 2009; Qin et al., 2012; Pêkala et al., 2014). There are few reports on the isolation and characterization of *S. putrefaciens,* a poorly known pathogen of freshwater fish from diseased loach (Qin et al., 2014).

In September 2014, a disease outbreak occurred in cultured loach *M. anguillicaudatus,* in central China. All diseased loaches presented with clinical symptoms of necrotic skin lesions and ulcers, hemorrhages at the base of pectoral fins, and caudal fins rotted with ragged edges. In this study, a Gram-negative bacterium was isolated from the diseased loaches, and identified as *Shewanella sp*. by 16S rRNA and gyrase B (gyrB) gene sequences. The pathogenicity was confirmed in loach through reinoculation. To our knowledge, this is the second report only, on the isolation and characterization of *Shewanella sp*. from cultured loach.

Materials and Methods

Isolation and culture of bacterial pathogen[s] from diseased loaches. An epizootic disease occurred in cultured loach (*M. anguillicaudatus*) at a farm at Xuzhou City, Jiangsu Province, China in September 2014. The diseased loaches (length 13±1cm) exhibited clinical signs of skin ulcers, pectoral fin base hemorrhages, and caudal fin rot, with ragged edges. Samples of skin, liver, and heart lesions of ten fish were streaked onto Luria-Bertani (LB) agar plates and incubated at 28℃ for 24h. Single colonies from plates were re-streaked on the same media to obtain pure growth isolates. Pure incubation of the isolate strain was stored at -70℃ in LB broth with a final concentration of 15% glycerol.

Physiological and biochemical tests. Morphological investigation was conducted using the gram-staining method. Biochemical tests were performed by using commercial microtest systems (Hangzhou Tianhe Microorganism Reagent Co., Ltd, China), including oxidase, ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase and urease; production of indole and H2S; reactions for Methyl red and Voges-Proskauer; nitrate reductase, gluconate and DNase; hydrolyzation of gelatin, starch, sodium hippurate, esculin; acid production from sorbitol and erythritol, lactose, arabinose, raffinose, sorbose, etc. The microtest tubes were incubated for 48h before reading the reactions.

16S rRNA gene and gyrB gene sequences analysis. Total genomic DNA of the isolate strain was extracted by using the UNIQ-10 column genomic DNA extraction kit (Sangon, China) according to manufacturer instructions. The almost complete 16S rRNA gene was amplified by PCR with one set of universal primer 27F: 5′- AGAGTTTGATCATGGCTCAG -3′ and 1492R: 5′- TACGGTTACCTTGTTACGACTT -3′ (Tm=55℃). The gyrB gene was amplified by PCR with a pair of specific primer gyrBF: 5′- AATTCTGGCAAAACGGGTGC -3′ and gyrBR: 5′- TGAGTCAGTGCTGGTTCGTC -3′ (Tm=53℃), which was designed by Primer-Blast software. PCR amplification was performed under the following conditions: an initial denaturation step at 95℃ for 2min; 30 cycles of 95℃ for 30s, annealing for 30s and 72℃for 1min; a final extension step at 72℃for 10 min. The PCR products were evaluated by eletrophoresis in 1% agarose gel by staining with ethidium bromide (Kumar et al., 2015).

The 16S rRNA and gyrB gene PCR products were sequenced by Sangon (China). The BLAST search was done at the National Center for Biotechnology Information (http://www.ncbi.nih.gov/BLAST/). Phylogenetic trees were constructed using the neighbor-joining algorithm of MEGA 5.1 software, with 5000 bootstrap replicates.

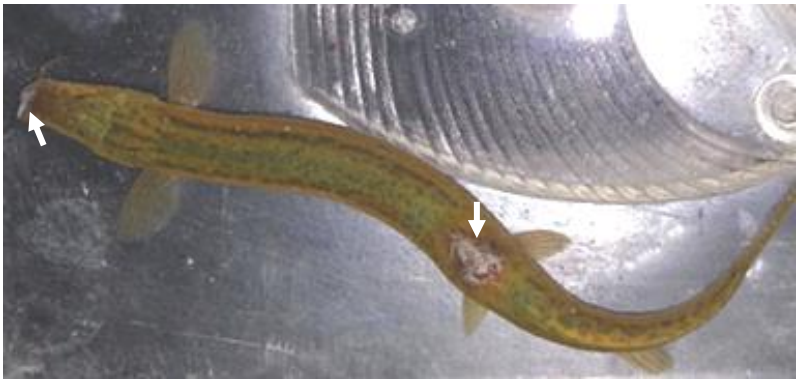
Experimental infection. Twenty healthy loaches, average length 13±1cm, were used for inoculation experiments after an acclimation period of 14 days. Loaches were randomly divided into two groups with ten loaches in each group. Water was maintained at 20±1℃. The infection group was inoculated by immersion infected with the MS1 strain at dosage concentration of approximately 2.0×10^8 CFU/ml, while the control group was untreated. Duration of experiment was 5 days.

Antimicrobial susceptibility test. The antimicrobial susceptibility tests of the isolate were conducted using Kirby-Bauer disc diffusion method on Mueller-Hinton agar plates. The antimicrobial agents (Hangzhou Microbial Reagent Co., Ltd, China) included ampicillin (10), amoxicillin (10), carbenicillin (100), meropenem (10), imipenem (10), cefamandole (30), cefixime (5), cefotaxime (30), cephalothin (30), cephalexin (30), cefoperazone (75), piperacillin (100), amikacin (30), gentamicin (10), kanamycin (30), netilmicin (30), neomycin (30), streptomycin (10), tetracycline (30), chloramphenicol (30), nitrofurantoin (300), norfloxacin (10), ofloxacin (5), pefloxacin (10), enrofloxacin (5), enoxacin (10), sulfamethoxazole/trimethoprim (23.75/1.25), sulphafurazole (300), trimethoprim (5), azithromycin (15), erythromycin (15), teicoplanin (30), vancomycin (30), rifampicin (5), clindamycin (2), lincomycin (2). The figures in brackets indicate the dose of each antibiotic (μg per disc).

Results

Isolation and biochemical characteristics of MS1 strain. One dominant strain was isolated from skin ulcerations of the diseased loaches (Fig. 1), and tentatively named strain MS1.

Fig. 1 Skin ulceration of diseased loaches infected by the isolate MS1. Arrows indicate local lesions.



The bacterial cells were Gram-negative bacilli. (see Fig. 2) Colonies on LB plate were opaque, circular, smooth, and convex, with an intact edge, measuring 0.8-1.0 mm in diameter after 24h incubation, at temperatures between 4–30℃. The isolate MS1 can grow in the presence of 1–4% NaCl (w/v), but cannot grow in a solution of more than 5% NaCl (w/v). Biochemical tests results are summarized in Table 1, the isolate MS1 which has a key phenotypic characteristic of sulfur reduction for *Shewanella sp.*, reacted positively for gelatin, oxidase, methyl red, urease and ornithine decarboxylase, and negatively for acids from galactose, maltose, sucrose, mannitol, erythritol, etc.

Fig. 2 The strain MS1 was stained as Gram-negative rod-shaped cells.

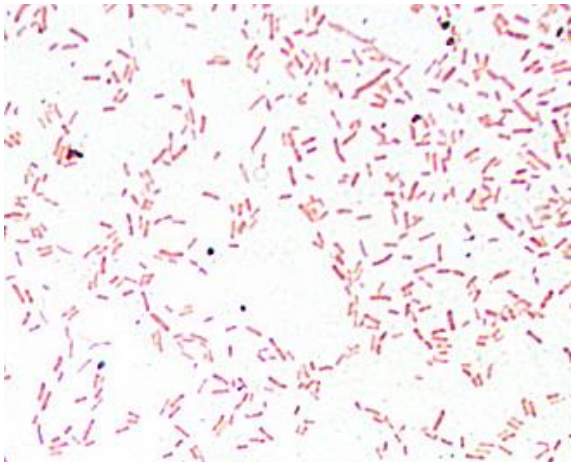


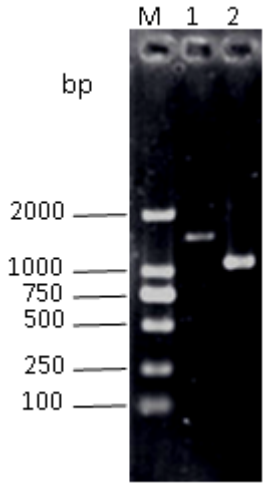
Table 1. Morphological and biochemical properties of the strain MS1 isolated from the diseased loaches.

Characteristics	*MS1*	*Characteristics*	*MS1*
Motility	+	Melibiose	-
Gelatinase	+	Fructose	-
Oxidase	+	Arabinose	-
Ornithine decarboxylase	+	Sucrose	-
Lysine decarboxylase	-	Rhamnose	-
Arginine dihydrolase	-	Sorbose	-
Methyl red	+	Galactose	-
Voges-Proskauer	-	Trehalose	-
Glucose (gas)	-	Cellobiose	-
ONPG	-	Raffinose	-
KCN growth	-	Xylose	-
O/129 growth	-	Mannose	-
Phenylalanine deaminase	-	Maltose	-
Nitrate reductase	+	Erythritol	-
H_2S production	+	Salicin	-
DNase	+	Adonitol	-
Glucosamine	-	Sorbitol	-
Gluconate	±	Xylitol	-
Citrate	-	Dulcitol	-
Malonate	-	Mannitol	-
Urease	+	Arabitol	-
Amygdalin	-	Inositol	-
Sodium hippurate	-	Growth	
Indole	-	4℃	+
Dextrin	-	30℃	+
Esculin	-	40℃	-
Starch	-	1% NaCl (w/v)	+
Acids from		4% NaCl (w/v)	+
Lactose	-	5% NaCl (w/v)	-
Glucose	-	6% NaCl (w/v)	-

+: positive. −: negative. ±: weak reaction.

16S rRNA and gyrB gene sequences analysis. The 16S rRNA and gyrB gene sequences of the isolate MS1 were 1507 bp and 1110 bp in length (Fig. 3), and were submitted to GenBank with an accession number of KM522840 and KM522841, respectively. The results of Blast alignments showed that the isolate MS1 shared the highest 16S rRNA sequence identities (98.93%, 98.87%) with the species of *Shewanella sp.* MR7 (CP000444) and *Shewanella sp.* MR4 (CP000446). The phylogenetic tree based on 16S rRNA sequences showed that the isolate MS1 is most closely related to the new strains *Shewanella sp.* MR7 and MR4 (Fig. 4). Furthermore, sequencing of MS1 gyrB gene also demonstrated the closest relationship with *Shewanella sp.* MR4 and *Shewanella sp.* MR7. The gyrB gene sequence from MS1 exhibited 89.64–81.40% identities to *Shewanella sp.* type strains.

Fig. 3 Agarose gel electrophoresis of PCR products of the 16S rRNA and gyrB gene of the isolate MS1. M: Marker. 1: 16S rRNA; 2: gyrB g



Experimental infection results. Through inoculation experiments the isolate MS1 was confirmed to be the pathogen in the infected loaches. Results indicated that the *Shewanella sp.* strain MS1 had a mortality rate of 100% at a dose of 2.0×10^8 CFU/mL. Clinical signs, lesions, and microscopic signs produced by experimental inoculation were similar to those observed in natural infections. The moribund loaches exhibited sluggish behavior, ulcers on the skin, and hemorrhages at fin base. There were no clinical signs in the control group.

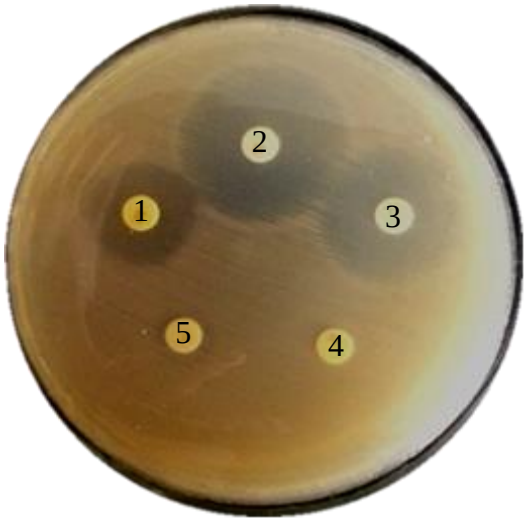
Antimicrobial susceptibility results. The results of three replicates of antimicrobial susceptibility tests showed that the isolate MS1 was susceptible to most antimicrobial agents tested including β-lactams (penicillins and cephalosporins), aminoglycosides, quinolones and sulphonamides, but resistant to glycopeptides (vancomycin, teicoplanin) and lincosamide (lincomycin, clindamycin) (Table 2; Fig. 5).

Table 2. Antimicrobial susceptibility patterns of the isolate MS1.

Antimicrobial agents	Sensitivity	Antimicrobial agents	Sensitivity
Ampicillin	23/S	Norfloxacin	29/S
Amoxicillin	24/S	Ofloxacin	23/S
Carbenicillin	21/S	Pefloxacin	30/S
Piperacillin	21/S	Enrofloxacin	31/S
Meropenem	21/S	Enoxacin	30/S
Imipenem	17/S	Sulphafurazole	20/S
Cephalothin	16/S	Sulfamethoxazole/trimethoprim	26/S
Cephalexin	20/S	Trimethoprim	23/S
Cefamandole	20/S	Azithromycin	29/S
Cefixime	25/S	Erythromycin	26/S
Cefoperazone	25/S	Chloramphenicol	32/S
Cefotaxime	29/S	Nitrofurantoin	17/S
Amikacin	22/S	Tetracycline	18/S
Gentamycin	20/S	Rifampicin	20/S
Kanamycin	17/S	Vancomycin	0/R
Netilmicin	25/S	Teicoplanin	0/R
Neomycin	19/S	Clindamycin	0/R
Streptomycin	19/S	Lincomycin	0/R

Data were represented mean of zone inhibition (mm) of three replicates. S: susceptible. R: resistant.

Fig. 5 Antimicrobial susceptibility testing of the strain MS1. 1: tetracycline; 2: azithromycin; 3: erythromycin; 4: vancomycin; 5: teicoplanin.



Discussion

The *Shewanella* genus belongs to the *Alteromonadaceae* family, found in various freshwater and marine environments (Pêkala et al., 2014; Qin et al., 2014; Toffin et al., 2004). *Shewanella spp.* have been identified as opportunistic pathogens in vertebrates, and have been considered dominant pathogens for clinical infections such as ulcers, bacteremia, and skin infections (Holt et al., 2005; Lee et al., 2016; Janda and Alameda, 2014). *Shewanella*-like isolates have been described as possible causative agents of lesions in some fish species (Subasinghe and Shariff, 1992; Cristóbal et al., 2008; Qin et al., 2014). Several new species of *Shewanella* have been identified using modern molecular methods (Qin et al., 2014). A new *Shewanella sp* strain MS1 was isolated from skin ulcers of diseased loaches *M. anguillicaudatus*, and was confirmed to be the causative agent responsible for 100% mortality. Compared with the isolate described by Qin et al. (2014), the *S. putrefaciens* strain from *M. anguillicaudatus* caused only 85% mortality. *Shewanella* infection has become a problem in cultured loach causing significant economic losses in China (Su et al., 2006; Li, 2009; Qin et al., 2014).

There is a high level of similarity of 16S rRNA gene sequences between *Shewanella* species (Venkateswaran et al., 1999; Cristóbal et al., 2008; Sekar et al., 2006). In this study, blast alignments showed that the isolate MS1 shared the highest 16S rRNA sequence identities of 98.93% and 98.87% with two new species of *Shewanella sp.* MR7 and *Shewanella sp.* MR4, followed by an identity of 98.73% to *S. putrefaciens* from the cultured loach, and 98.13%, 97.60% to *S. xiamenensis* and *S. oneidensis*, respectively. The phylogenetic tree constructed on 16S rRNA sequences indicated that the strain MS1 was most closely related to latest listed *Shewanella spp.* from aquatic environments. Phylogenetic analysis grouped the MS1 strain in the *Shewanella sp.* cluster, since their sequence similarity was more than 98% with two new reference strains MR4 and MR7. Two known genome sequences data were obtained from the Department of Energy, Joint Genome Institute (http://www.jgi.doe.gov), which are available with 4084, 4178 predicted gene encoding 3924, 4006 proteins for *Shewanella sp.* strains MR4 and MR7 genome, respectively. The phylogenetic relationship of *Shewanella sp.* MR4, *Shewanella sp.* MR7 with other typical *Shewanella* species based on 16S rRNA showed that these new species formed a cluster together with *Shewanella sp.* (Kan et al., 2011). The gyrB gene has been used in phylogenetic studies in members of *Pseudomonas*, *Vibrio,* and *Shewanella* genera (Yamamoto et al., 2000; Parvathi et al., 2005; Cristóbal et al., 2008). One pair of specific primers was designed for amplifying MS1 gyrB gene, and the phylogenetic tree derived from the gyrB gene sequences was similar to the 16S rRNA tree (data not shown). Several distinct characteristics related to acid production and growth temperature (Huang et al., 2010; Pêkala et al., 2014) of the isolate MS1 were observed with other species of *Shewanella* such as *S. putrefaciens, S. xiamenensis and S. oneidensis*. The MS1 strain was able to grow in 1-4% NaCl indicating that the isolate can adapt to a freshwater environment. It was identified as *Shewanella sp.* by

physiological and biochemical characteristic analysis. Results of this study will contribute to understanding the *Shewanella sp.* phenotype characteristics and provide a reference for identification of *Shewanella* species in fish.

Antimicrobials have been recognized as potential options for treatment of bacterial infection caused by *Shewanella* spp., both in aquaculture and in human beings (Qin et al., 2014; Holt et al., 2005; Vignier et al., 2013). Most *Shewanella* spp. are susceptible to commonly used antimicrobial agents, particularly third- and fourth-generation cephalosporins, piperacillin, ciprofloxacin, and gentamicin (Vignier et al., 2013; Holt et al., 2005). As human pathogens, *S. algae* and *S. putrefaciens* were susceptible to aminoglycosides, carbapenems, and quinolones. They also displayed variable susceptibilities to β-lactams (penicillins and cephalosporins) (Heritier et al., 2004; Janda and Alameda, 2014; Pêkala et al., 2014). It is important to note that antimicrobial susceptibility patterns of isolate MS1 from diseased fish are similar to those from humans (Vignier et al., 2013; Janda and Alameda, 2014). *Shewanella* can show resistance to glycopeptides and lincosamide, although the isolate MS1 was susceptible to all antimicrobial agents tested in the present study. We concluded that β-lactams (provided that the strain is susceptible), aminoglycosides and quinolones may be the drugs of choice in treatment of infections caused by *Shewanella sp.* in loach.

Shewanella is usually collected from marine environments, and rarely from freshwater fish. A new *Shewanella sp.* strain MS1 was isolated from diseased loaches in central China. The results obtained both with molecular methods by sequencing 16S rRNA and gyrB genes, and phenotype testing for identification of *Shewanella* species, contributed to the understanding of *Shewanella sp.* phenotype characteristics. The isolate MS1 was confirmed as the pathogen in loaches. We believe that this report can provide a scientific reference for characterization of *Shewanella* sp., and prevention of bacterial disease in fish.

Acknowledgements

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