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Isolation, identification and characterization of *Shewanella algae* from reared tongue sole, *Cynoglossus semilaevis* Günther

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ABSTRACT

In this study, a Gram-negative bacterium was isolated from diseased *Cynoglossus semilaevis* (*C. semilaevis*) and tentatively named strain CSG-15. It was preliminary identified as *Shewanella algae* (*S. algae*) based on morphological, physiological and biochemical characteristics. The sequenced 16S rRNA gene of CSG-15 strain (Genbank accession no. KX455851) was 1509 bp. And it exhibited 99.86%, 99.86% and 99.80% of identities compared with those of *S. algae* strains ATCC51192 (NR117771), YJ06114 (EF542799), and MAS2737 (GQ372875), respectively. Phylogenetic analysis grouped CSG-15 strain in the *S. algae* cluster, and showed the closest relation to *S. algae* ATCC51192 and YJ06114. Infection experiment showed that the median lethal dosage (LD_{50}) for CSG-15 was calculated as 1.0×10^6 CFU/g fish weight. The pathological observation on the intestine showed that CSG-15 strain caused noticeable histological lesions, such as inflammatory cell infiltrating and villi shedding. Antimicrobial susceptibility test showed that it was highly sensitive to most of aminoglycosides, β-lactams, tetracyclines, quinolones, macrolides aminocyclitol, sulphonamides, chloramphenicols antibiotic. Growing characteristics showed that CSG-15 could grow under the condition of temperature 26–34 °C, pH 5–9 and 20–50 ppt, and had a better adaptability to environment. The growth curve of strain CSG-15 under the condition of 30 °C, pH 7.0 and 30 ppt showed four distinct phases of bacterial growth. To the best of our knowledge, this is the first report of *S. algae* linked to diseased *C. semilaevis*.

Statement of relevance: In this study, a new *Shewanella algae* strain was isolated from diseased *C. semilaevis* for the first time. It was identified based on morphological, physiological, biochemical characteristics and 16S rRNA gene sequences analysis. Subsequently, antimicrobial susceptibility, pathogenicity and growing characteristics of *S. algae* were studied. This report can provide a scientific reference for characterization of Shewanella species, prevention and treatment of bacterial disease caused by *S. algae* in fish.

1. Introduction

The tongue sole *Cynoglossus semilaevis* (*C. semilaevis*) Günther, a demersal spared fish, is widely distributed in the coast of China, especially in the Bohai Sea and Huanghai Sea (Wang et al., 2015). Upon the successful establishment of the breeding method, this piscine species has become one of the most attractive candidates used in aquaculture. The high fillet yield and flesh quality represent major factors contributing to attractive market values. With rapid development of industrial culture in recent years, various infectious diseases in *C. semilaevis* have emerged and caused great economic losses. Recently, some bacterial pathogens have been isolated from diseased *C. semilaevis*, including *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Listonella anguillarum*, *Photobacterium damselae* (Chen et al., 2012; Hu et al., 2014; Wang et al., 2007; Zhang et al., 2009).

Shewanella, a new genus belonging to Shewanellaceae, is widely distributed in seawater and sediments (Ivanova et al., 2004; Satomi et al., 2007; Xiao et al., 2007). Some species of Shewanella are conditionally pathogenic bacteria to aquatic animals and human (Yang et al., 2009). Most of the clinical isolates are *Shewanella putrefaciens* (*S. putrefaciens*), but recent data suggest that many of these isolates should be classified as the genetically distinct species *Shewanella algae* (*S. algae*) (Holt et al., 2005). Some argue that *S. algae* may be a more virulent species and the most important pathogen in Shewanella (Khashe and Janda, 1998). *S. algae* usually infects ears and soft tissues of humans in clinics, and causes more serious infections, such as bacteremia, peritonitis, neonatal sepsis and pyogenic flexor tenosynovitis (Charles et al., 2015; Fluke et al., 2016; Kim et al., 2014). Recently, this pathogen has also been isolated from aquatic animals including *Scinenops ocellata*, marine sponge *Callyspongia diffusa*, Babylonia sp. (Chen et al., 2003; Li et al., 2015; Rachanamol et al., 2014; Zhang et al., 2013).

However, to the best of our knowledge, *S. algae* isolated from *C. semilaevis* have never been reported in the literature. In this study, a Gram-negative bacterium was isolated from diseased *C. semilaevis*,

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and identified as *S. algae* based on physiological and biochemical characteristics and 16S rRNA gene sequence analysis. The results of isolation and characterization of the *S. algae* strain and its growing characteristics were presented in this paper.

2. Materials and methods

2.1. Bacterial isolation

Samples obtained from liver, kidney and ascites of diseased and moribund *C. semilaevis* with symptoms of ascitic disease ($n = 10$, 120–140 g) were streaked by sterile swabs onto 2216E nutrient agar plates (10 g/L tryptone, 5 g/L yeast extract powder, 15 g/L NaCl, 0.01 g/L ferric phosphate and 15 g/L agar; pH 7.5), and incubated at 30 °C for 24 h. 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 −80 °C in 2216E broth (10 g/L tryptone, 5 g/L yeast extract powder, 15 g/L NaCl and 0.01 g/L ferric phosphate; pH 7.5) at a final concentration of 15% (v/v) sterile glycerol.

2.2. Biochemical characteristics tests

Morphological investigation was done using the gram-staining method. Biochemical tests were performed using commercial microtest systems (Hangzhou Tianhe Microorganism Reagent Co., Ltd., China), including Oxidative/Fermentative, Voges Proskauer test, Methyl Red test, urea, gluconate, H_2S, citrate, indole, oxidase, catalase, lysine, arginine, ornithine, NO_3^- reductase, adonitol, saligenin, sorbitol, phenylalanine, mannose, sucrose, xylose, raffinose, glucose, motility. Growth condition at 0%–10% of NaCl (w/v) and 4–42 °C was also studied. The incubation was performed at 30 °C for 48 h and the results were observed.

2.3. 16S rRNA gene sequences analysis

Total genomic DNA of the isolate strain for the PCR was extracted using the Ezup Column Bacteria Genomic DNA extraction Kit (Sangon, China). The 16S rRNA gene was amplified by PCR with one set of universal primer (Cao et al., 2007), 7F: 5′-CAGAGTTTGATCCTGGCT-3′ and 1540R: 5′-AGGAGGTGATCCAGCCGCA-3′. Amplification conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR amplification products were checked on 1% agarose gels by staining with ethidium bromide and purified with a SanPrep Column DNA Gel Extraction Kit (Sangon, China). A pMD-T vector was used for cloning the PCR products according to the instructions of the manufacturer (Takara, Japan). Plasmid DNA was isolated from *E. coli* with a SanPrep Column Plasmid Mini-Preps Kit (Sangon, China) and sequenced with 3730XL DNA Analyzer (Applied Biosystems, America). The BLAST search was done at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nih.gov/BLAST/). Phylogenetic trees were constructed using the neighbor-joining algorithm of MEGA 5.1 software, with 5000 bootstrap replicates.

2.4. Experimental infections

C. semilaevis was provided by Haifa Aquaculture Co., Ltd., Tianjin, China. Fish were transferred back to Tianjin Agricultural University and maintained at optimal rearing condition for one week, with water temperature adjusted to 23 °C, pH 7.5 and salinity 22 ppt. Aeration was provided to maintain optimal DO and fish were fed with commercial pellets formulated by the company twice daily.

Forty-eight healthy *C. semilaevis* with a mean weight of 35.5 ± 3 g and length of 19 ± 0.5 cm were used for challenge experiments. *C. semilaevis* were divided into six groups with eight fish in each group. Five groups were injected intraperitoneally with the suspension of a dominant strain tentatively named CSG-15 at a concentration of 1×10^6 CFU/mL, 1×10^7 CFU/mL, 1×10^8 CFU/mL, 1×10^9 CFU/mL and 1×10^{10} CFU/mL, respectively. The dose for the infection was 200 μL/fish. And the last group used as control was injected with a same dose of 0.9% physiological saline. After injection, the health condition and mortality of *C. semilaevis* were observed for 21 days. Pathogenicity was confirmed by the occurrence of disease, including clinical signs and morbidity congruent with those of natural cases, re-isolation and re-identification of the tested bacteria from liver, kidney and ascites of experimentally infected fish.

2.5. Histopathology

The intestine tissue of the infected fish with the clinical signs was fixed in Bouin's fixative during 24 h at room temperature. Serial sections of paraffin embedded tissues of 5 μm thicknesses were cut using a microtome (Thermo, America) and stained with hematoxylin and eosin (H&E) for further examination under light microscope (Leica, Germany). And the intestine tissue of the fish injected with physiological saline was used as control.

2.6. Antimicrobial susceptibility test

The antimicrobial susceptibility tests for the isolate were done using Kirby-Bauer disc diffusion method on 2216E nutrient agar plates. The antibiotic impregnated discs (Hangzhou Tianhe, China) included minocycline (30 μg), doxycycline (30 μg), tetracycline (30 μg), erythromycin (15 μg), neomycin (30 μg), gentamicin (100 μg), kanamycin (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), compound sulfamethoxazole (23.75/1.25 μg), ciprofloxacin (5 μg), aboren (30 μg), streptomycin (10 μg), amoxicillin (10 μg), amikacin (30 μg), cefepime (30 μg), tobramycin (10 μg), polymyxin B (300 μg), cefalexin (30 μg), cefuroxime (30 μg), cefradine (30 μg), cefoperazone (75 μg), cefazolin (30 μg), ampicillin (10 μg), oxacillin (1 μg), carbenicillin (100 μg), piperacillin (100 μg), penicillin (10 μg), clarithromycin (15 μg), norfloxacin (10 μg), aztreonam (30 μg), cefotaxime (30 μg), clindamycin (2 μg), cephalothin (30 μg), vancomycin (30 μg), spectinomycin (100 μg), ofloxacin (5 μg), chloramphenicol (30 μg) (Table 1). The diameter of inhibition zone below 13 mm was regarded as resistant, between 13 mm and 19 mm moderately susceptible, and above 19 mm susceptible (Zhang et al., 2010).

2.7. Growing characteristics

CSG-15 was subcultured twice successively (24 h at 30 °C) in 2216E broth before inoculation for determination. A modified basic medium (MBM) was prepared, supplemented with tryptone 10 g/L, yeast extract powder 5 g/L and ferric phosphate 0.01 g/L. The medium was then dispensed in 50 mL portions into 250 mL Erlenmeyer flasks.

For each combined condition, the temperature, pH value and NaCl concentration were adjusted based on MBM. The effect of pH on the isolate growth was studied at 5, 6, 7, 8 and 9 in MBM with 30 ppt at 30 °C; the effect of salinity at 20 ppt, 30 ppt and 50 ppt, were studied in MBM, with pH value of 7.0 at 30 °C; the effect of temperature was studied at 26 °C, 30 °C and 34 °C in MBM with pH value of 7.0 at 30 ppt. Before sterilized by autoclaving, the pH was adjusted to the desired values with NaOH (1 mol/L) or HCl (1 mol/L). The target salinity values were obtained from the high salinity MBM medium (50 g/L) by appropriate dilution with MBM. All flasks were inoculated with 0.2 mL of bacterial suspension ($OD_{600} = 0.1$) and cultured at 180 rpm and then growth was monitored for 28 h by measuring the optical density (OD) at 600 nm every 2 h.