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Isolation, identification and physiological growth characteristics of *Pseudomonas parafulva* from diseased *Acipenser baerii*

Abstract. The article presents the results of isolation of the bacterial pathogen *Pseudomonas parafulva* from a diseased Siberian sturgeon *(Acipenser baerii)* grown in a recirculating aquaculture system. The studied diseased individuals of the Siberian sturgeon *(Acipenser baerii)* were characterized by reduced activity, did not consume compound feed, on the body of some individuals there were hemorrhages at the bases of the fins, as well as deep penetrating ulcers. Internal organs (liver, spleen and kidneys) and washes from open ulcers were used to isolate the bacterium. As a result of studies of biological materials, the dominant bacterium was used for further identification. The bacterium is characterized as a gram-negative, motile oxidase-positive rod, in the Voges-Proskauer and methyl red test negative, in the O/F test it is characterized as oxidative. As a result of molecular genetic analysis of the 16S rRNA gene sequence of the isolated bacterium, 99% identity with other strains of *P. parafulva* was determined. Subsequently, the isolated strain was named AB004, and the 16S rRNA sequence was registered in the National Center for Biotechnology Information (NCBI) database under registration number OK634400. According to the results of the physiological characteristics of AB004, the optimal indicators of cultivation in the Luria-Bertani (LB) medium are: NaCl concentration – 1%, pH value – 7.0, temperature – 37°C.

Keywords: recirculating aquaculture system, *Acipenser baerii*, pseudomonosis, *Pseudomonas parafulva*, 16S rRNA gene.

Introduction

The annual reduction of sturgeons in natural conditions contributed to the development of industrial aquaculture of these valuable species. The main reasons for the significant reduction are: deterioration of the general ecological situation of natural habitats, including changes in the water regime, hydrochemical composition, and also poaching [1, 2]. It is for the purpose of restoring the natural numbers of sturgeon populations that cultivation and artificial reproduction are carried out in facilities with a recirculating aquaculture system, where all optimal growing conditions are created [3]. One of the valuable objects of sturgeon breeding is the Siberian sturgeon *(Acipenser baerii)*. In the world sturgeon aquaculture, the Siberian sturgeon *(Acipenser baerii)* occupies a leading position, as it is characterized by a fast growth rate and significantly early puberty (5–6 and 6–8 years for male and female respectively) among sturgeon species [4, 5].

But despite the creation of optimal growing conditions in industrial aquaculture, there is a risk of disease in sturgeons caused by bacterial pathogens of the genus *Pseudomonas*. The following representatives of the *Pseudomonas* genus are the main bacteria that are of a massive nature infecting fish in aquaculture: *P. aeruginosa*, *P. fluorescens*, *P. putida* [6–8]. Bacteria of the genus *Pseudomonas* are the causative agents of pseudomonosis disease affecting sturgeons *(Acipenseridae)*, salmonids *(Salmonidae)* and cyprinids *(Cyprinidae)* [9, 10]. Hemorrhagic septicemia and ulcers, as well as clouding of the eyes, are observed in fish as a result of damage by bacteria of the genus *Pseudomonas* [11]. Mortality of fish as a result of diseases caused by bacteria of the genus *Pseudomonas* can reach 100% [12].

P. parafulva is characterized as a Gram-negative, motile, non-spore forming bacterium. Able to live in a wide temperature range from 4 to 37°C, a taxonomically close species of *Pseudomonas putida* [13].

Materials and methods

The study used diseased sturgeons *(Acipenser baerii)* reared in conditions of recirculating aquaculture systems (RAS), Uralsk, Kazakhstan. Diseased fish were transported in the microbiological laboratory of Zhangir Khan West Kazakhstan Agrarian Technical University. The fish were examined for the presence of external parasites. On the body of diseased fish were found ulcers with deep penetrating muscle necrosis, hemorrhages on the fish body and in the pelvic fins, branchial ischemia, and inflammation of the anus.

To isolate bacterial pathogens, biological materials of internal organs and washes from ulcers on the fish body were selected. To isolate bacteria, Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar) was used at pH 7.0 [14]. Cultures of bacteria were grown at a temperature of 30°C within 24 hours. Dominant colonies of bacteria were selected for further research. A single colony of bacteria was inoculated into LB medium and grown for 18 hours at 37°C. The isolated bacterial culture was preserved on LB agar at 4°C and in LB at – 75°C with the addition of sterile glycerol 50% (v/v).

Analysis of the physiological and biochemical properties of AB004 was carried out with the following tests shown in Table 1. Identification media (Condalab, Spain; TM Media, India) were used for the analysis. Results were observed after incubation according to the manufacturer's instructions.

To isolate total DNA, an overnight bacterial culture cultivated at 37°C in LB medium. 400 µl of an overnight culture of bacteria was taken into 0.5 ml Eppendorf tubes, centrifuged at 6000 prm for 5 minutes. The resulting cell pellet was resuspended in 200 µl of autoclaved distilled water, then boiled at 100°C in a thermo bath for 10 minutes. The cell culture after boiling was centrifuged at 20000 g for 10 minutes at 5°C. The resulting supernatant was used as the total DNA of the studied bacterial culture. The obtained DNA was processed using nanodrop (Thermo Scientific). The obtained bacterial DNA was stored in a freezer at – 20°C [15]. To amplify the 16S rRNA gene, we used universal bacterial primers 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGCTACCTTGTTACGACTT-3' [16]. The studied amplicons were sequenced by Biofidal (Vaulx-en-Velin, France; http://www.biofidal-lab.com). BLAST sequence searches via the NCBI website and phylogenetic tree was constructed by the neighbor-joining method in the MEGA XI software according to Han et al. (2017) [17].

To study antibiotic resistance, commercial discs (Condalab, Spain) were used, which included 19 antibiotics with a concentration from 1 to 300 µg (Table 2). To determine antibiotic resistance, Mueller-Hinton agar was used and incubated with AB004 at 35°C for 24 hours [18].

To determine the growth characteristics of AB004, the following factors were selected: pH (3.0, 5.0, 7.0, 9.0), temperature (27, 32, 37, 42°C) and concentration of NaCl (0-5%) [19]. Strain AB004 was grown in LB medium until the optical density of the culture was reached at OD_{600} equal to 1.0. This suspension (0.5 ml) was used for inoculation and growth measurement. Incubation was carried out on a shaker at 150 rpm, cell growth was determined by the change in the optical density (OD_{600}) of the culture every hour for 10 hours. All experiments were carried out in triplicate.

Results and discussion

AB004 is a gram-negative, motile bacterium capable of growing in LB medium with NaCl concentration of 0-5%, pH 7.0-9.0 at 27-42°C (Table 1).

Table 1 – Biochemical characteristics of AB004

#	Characteristics	Reaction	#	Characteristics	Reaction
1	Gram stain	-	18	D-xylose	+
2	Morphology	rod	19	Lactose	-
3	Motility	+		Growth under conditions:	
4	Oxidase	+	20	0% NaCl	+
5	Methyl red	-	21	1% NaCl	+
6	Voges-Proskauer test	-	22	2% NaCl	+
7	O/F test	O	23	3% NaCl	+
8	Hydrolysis of gelatin	-	24	4% NaCl	+

Table continuation

#	Characteristics	Reaction	#	Characteristics	Reaction
9	Hydrolysis of esculin	-	25	5% NaCl	+
10	H_2S formation	-	26	4°C	+
11	Indole formation	+	27	27°C	+
12	Lysine decarboxylase	-	28	32°C	+
13	Ornithine decarboxylase	-	29	37°C	+
14	Arginine dihydrolase	+	30	42°C	+
15	ONPG	-	31	pH 3.0	-
Acid formation from:			32	pH 5.0	-
16	Sucrose	-	33	pH 7.0	+
17	Trehalose	-	34	pH 9.0	+
Note: "+" – positive; "-" – negative; "O" – oxidative					

The strain showed a positive reaction to oxidase, indole formation, arginine dihydrolase, and also forms acid from D-xylose. A negative reaction was observed in tests for lysine and ornithine decarboxylase, H_2S formation, ONPG, sucrose, trehalose, lactose. AB004 is not capable of hydrolyzing gelatin and esculin. According to the results of the O/F test, the bacterium was identified as oxidative. In terms of the main biochemical characteristics, the isolated strain AB004 is relatively similar to the previously studied *P. putida* MTCC 7525 isolated from soil samples in India [20]. Thus, it was determined that *P. parafulva* is not only taxonomically but also biochemically similar to the bacterium *P. putida* [13].

The results of the AB004 antibiotic resistance analysis are presented in Table 2.

Table 2 – Sensitivity of AB004 to different antibiotics

Group	Antibiotic	Disk Content (μg)	AB004	
			Sensitivity	Zone diameter (mm)
Penicillins	Oxacillin	1	R	0
	Penicillin G	10	R	0
	Ampicillin	10	R	0
	Amoxicillin	10	R	0
Quinolones	Enrofloxacin	5	R	15.5±0.3
	Norfloxacin	10	S	25±0.7
Cephalosporins	Cefazolin	30	R	0
Aminoglycosides	Gentamicin	10	S	16.3±0.4
	Streptomycin	10	I	13.2±0.2
Nitrofurans	Nitrofurantoin	300	R	0
Tetracyclines	Tetracycline	30	I	15.5±0.3
	Oxytetracycline	30	R	14.5±0.3
Macrolides	Erythromycin	15	R	7.6±0.4
Lincomycins	Lincomycin	10	R	0
Rifamycins	Rifampicin	5	R	10.7±0.4
Coumarins	Novobiocin	30	R	0
Amphenicols	Chloramphenicol	10	R	8±0.7
	Florfenicol	30	R	11.3±1.6
Folic acid synthesis inhibitors	Trimethoprim + sulfamethoxazole	25	R	0
Note: R – resistant, I – intermediate, S – sensitive				

AB004 was found to be resistant to oxacillin, penicillin G, ampicillin, amoxicillin, enrofloxacin, cefazolin, nitrofurantoin, oxytetracycline, erythromycin, lincomycin, rifampicin, novobiocin, chloramphenicol, florfenicol, trimethoprim + sulfamethoxazole. Antibiotics were found to be sensitive for AB004: norfloxacin and gentamicin. Medium sensitivity was observed for streptomycin and tetracycline.

As a result of antibiotic resistance studies, it was determined that the isolated bacterium *Pseudomonas parafulva* AB004 is characterized as a multi-resistant strain that has shown resistance to 15 out of 19 antibiotics studied. The strain showed moderate resistance to streptomycin and tetracycline. Gentamicin and norfloxacin were found to be sensitive antibiotics for the isolate of *Pseudomonas parafulva*. So, it is generally known that the antibiotics gentamicin and norfloxacin have a strong bactericidal effect on a wide range of gram-negative bacterial pathogens, which include *Pseudomonas* spp., *Enterobacter* spp. and *Shigella* spp. [21, 22]. Thus, as a result of the study of antibiotic resistance, it is possible to use norfloxacin and gentamicin to inactivate the isolated strain of *Pseudomonas parafulva*.

The 16S rRNA sequence was 1457 bp in length with GenBank accession number OK634400 (Figure 1). In the analysis, 99.38-99.52% identity was observed with strains *Pseudomonas parafulva* (KX345930.1), *Pseudomonas parafulva* strain PRS09-11288 (CP019952.1), *Pseudomonas parafulva* JCM 11244 (LC507438.1). Based on the results obtained, phylogenetic trees were built to determine the relationship with other representatives of *Pseudomonas* spp. (Figure 2).

As a result of the study of the sequenced region of the 16S rRNA gene, it was determined that the isolated bacterium is identified as a representative of the genus *Pseudomonas*, the species *Pseudomonas parafulva*.

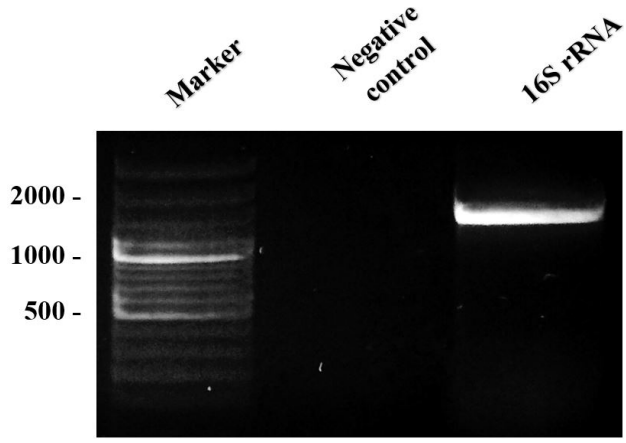

Figure 1 – Agarose gel electrophoresis of PCR product of the 16S rRNA gene of isolate *P. parafulva*

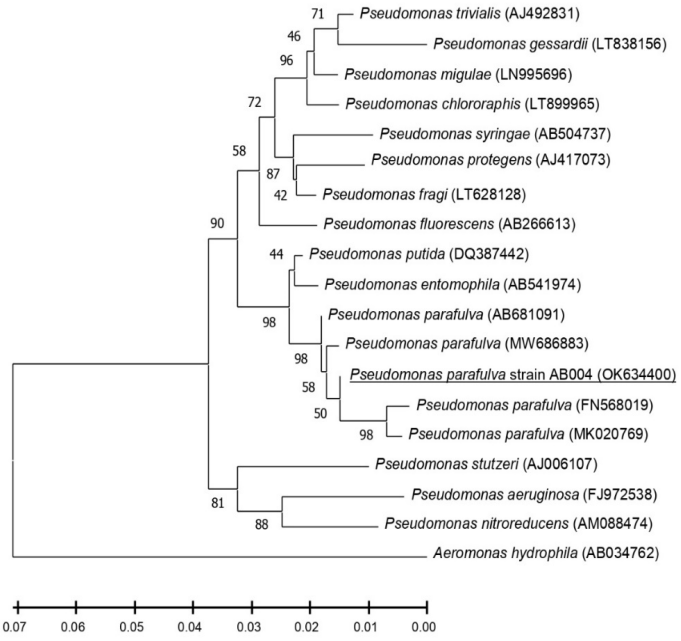

Figure 2 – Unrooted neighbor-joining phylogenetic tree based on 16S rRNA gene

According to the results of the studies of the growth characteristics, it was determined that the optimal indicators are pH 7.0, 1% NaCl and a temperature of 37°C (Figure 3). At the same time, it is noted that the AB004 strain is capable of growth under conditions of pH 7.0-9.0, 0-5% NaCl and 27-37°C.

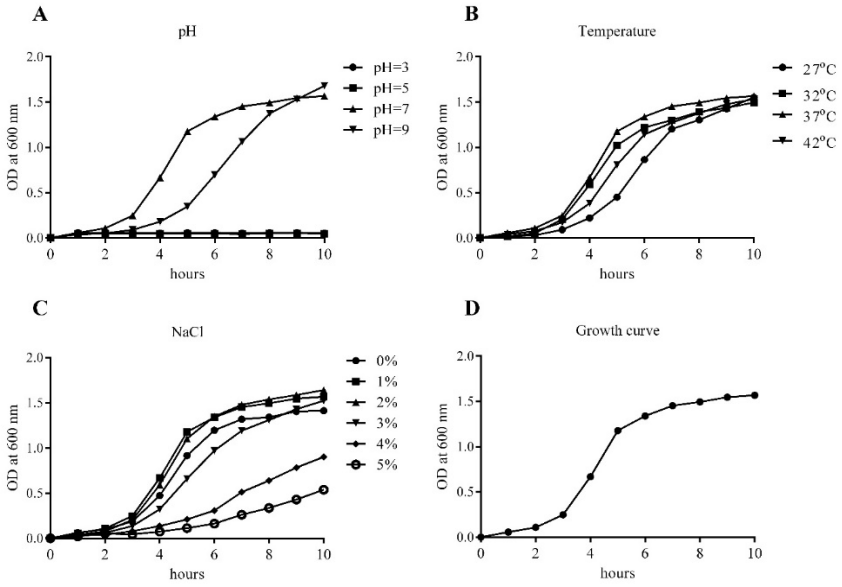

Figure 3 – Growing characteristics of AB004. A. Growth at pH 3-9, B. Growth at temperature 27-42oC, C. Growth at 0-5% NaCl, D. The growth curve of *P. parafulva* at pH 7.0, 37oC and 1% NaCl

As a result of the research, it was determined that the logarithmic phase of growth is observed in the time interval from 3 to 5 hours from the start of bacterial inoculation. Bacterial growth is observed at pH 7, 9, but at pH 9 there is a noticeable slowdown in the onset of logarithmic bacterial growth within 2 hours. At pH values of 3, 5, bacterial growth was not observed. The influence of temperature parameters on the growth of bacteria was insignificant, at temperatures of 27, 32, 37°C the growth curves were almost identical. A slight delay in the onset of logarithmic growth was observed at 42°C. Also, at a concentration in a medium with a NaCl content of 0-3%, the differences in growth curves are not large, as for concentrations of 4, 5%, the lag phase of bacteria increases to 3 hours. Thus, as a result of studies of the physiological characteristics of bacterial growth, it was found that the most optimal conditions for *P. parafulva* growth are pH 7, NaCl 1%, 37°C.

Conclusion

In sum, the bacterium *P. parafulva* was isolated and identified from the diseased Siberian sturgeon *(Acipenser baerii)* grown in industrial aquaculture. According to the results of the determination of antibiotic resistance, AB004 is characterized as a multidrug-resistant strain. The obtained results of biochemical tests, antibiotic resistance, as well as physiological characteristics of growth will serve as useful information about the bacterial pathogen of sturgeon *P. parafulva* aimed at implementing methods to combat the bacterium *P. parafulva*, which is one of the causative agents of sturgeon pseudomonosis.

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