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Original Research Articles

Isolation and identification of intestinal cellulolytic bacteria from red swamp crayfish (*Procambarus clarkii*)

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Red swamp crayfish (*Procambarus clarkii*) like to eat aquatic plants. The intestinal microorganism plays an important role in cellulose degradation and utilization. Isolating bacteria which can degrade cellulose from the intestines of *P. clarkii* can provide a theoretical basis for the development of probiotics in forage for *P. clarkii*. Three selective media including carboxymethyl-cellulose, microcrystalline cellulose, and cellobiose were used in plate cultures for 48h and then dyed with Congo red. It was found that 5 strains produced hydrolytic rings on 3 culture media at 48h. Based on the results of 16S rRNA molecular analysis, strains C, E, G, H, and M were identified as *Citrobacter sp.*, *Staphylococcus sp.*, *Acinetobacter johnsonii*, *Shewanella sp.*, and *Aeromonas caviae*, respectively. Specifically, *Staphylococcus sp.* exhibited the strongest capacity for the degradation of cellulose. *Shewanella sp.* showed the strongest degradation capacity for cellobiose. *Acinetobacter johnsonii* and *Shewanella sp.* can degrade cellulose and are expected to be used as probiotic feed for *P. clarkii*. This study can provide a theoretical basis for the healthy culture of *P. clarkii*.

INTRODUCTION

Cellulose is an important component of plant cell wall and it is present in a relatively large content in raw materials of vegetable-based feeds. Herbivorous grass carp (*Ctenopharyngodon idellus*) and blunt snout bream (*Megalobrama amblycephala*) digest cellulose from aquatic plants depending on the cellulose produced by the cellulolytic bacterial community in their intestinal tracts.[1-3] Cellulase is the generic term for a group of complex enzyme systems. It is mainly divided into three types based on function: 1) β-1,4-endoglucanases, which act on the non-crystalline region inside the cellulose, hydrolyses β-1,4 glucosidic bonds randomly, cuts long-chain cellulose molecules and produces abundant cellulose with non-reduced terminal micromolecules; 2) cellobiohydrolases, which act on the terminal of cellulose linear molecules, hydrolyses β-1,4 glucosidic bond, and cleaves cellobiose molecules. Hence, it is also known as cellobiose hydrolase; 3) β-glucosidases, which generally hydrolyses cellobiose or soluble cellodextrin into glucose molecules.[4,5]

Several microorganisms form complex communities in animal digestive tracts, resulting in a diverse ecosystem.[6] Intestinal microorganisms form a symbiotic relationship with the host.[7] They play an important role in nutrient absorption, growth, metabolism, host resistance and immune response to disease.[8-11] Several cellulolytic bacteria have been separated successfully from the intestinal tracts of grass carp, including *Aeromonas, Enterococcus, Bacillus, Enterobacterium,* and *Citrobacter sp.*[12-15]

Red swamp crayfish (*Procambarus clarkii*) is an important aquaculture shellfish in China. Between 2007 and 2022, the aquaculture output of *P. clarkii* in China increased from 265,500 tons to 2.8907 million tons. In 2022, the commercial value of *P. clarkii* industry in China was about 458 billion yuan. As omnivores, *P. clarkii* prefers aquatic plants and their intestinal tracts degrade cellulose, which might be related to the endogenous cellulose[16] and the presence of cellulose-degrading bacteria in the intestine. Feng et al.[17] screened cellulolytic bacteria, including *Bacillus velezensis* and *B. subtilis*, from the intestinal tracts of *P. clarkii* via pure culture method using a carboxymethylcellulose medium. There are few studies evaluating the cellulase activities of *P. clarkii* by using microcrystalline cellulose and

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cellobiose medium. In the present study, cellulolytic bacteria which can produce multiple cellulase types were obtained by screening from the intestines of *P. clarkii* using carboxymethylcellulose, microcrystalline cellulose and cellobiose. It provides a theoretical reference to develop probiotics in the feed supplied to *P. clarkii*.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Twelve healthy red swamp crayfish (20.32±0.95g) were obtained from a local farm in Changde, Hunan Province, China in June. The ratio of male to female was 1:1.

PREPARATION OF MEDIUM

Three media were prepared as described by Li et al.[2] The carboxymethyl-cellulose(CMC)-agar medium (1L) is composed of 10 g CMC, 4 g KH_2PO_4, 4 g Na_2HPO_4, 2 g tryptone, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.001g $CaCl_2$, 0.001g $FeSO_4{\cdot}7H_2O$, and 15 g agar powder. The microcrystalline cellulose (MC)-agar *medium* (1L) is composed of 10 g microcrystalline cellulose, 4 g KH_2PO_4, 4 g Na_2HPO_4, 2 g tryptone, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.001 g $CaCl_2$, 0.001g $FeSO_4{\cdot}7H_20$, and 15 g agar powder. The cellobiose (GC)-agar medium (1L) comprises 4 g esculoside, 1g ammonium ferric citrate, 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar powder. The tryptic soy agar (TSA) medium uses the TSA powder at a 40g /L mixing concentration.

SCREENING OF CELLULOLYTIC BACTERIA

Intestinal tracts of 12 *P. clarkii* samples were extracted under aseptic condition. The intestinal tracts and sterile PBS (0.1 mol/L, pH 7.2) were homogenised at the ratio of 1:9. The intestinal-PBS mixture was obtained after homogenization. The mixture was processed via serial dilution:10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Each dilution contained two preparations and was coated onto the TSA plate, followed by incubation at 28°C for 48 h. Several single colonies were selected for streaking on the TSA plate, and cultured for 48 h at 28°C. After purification three times, the bacteria were inoculated onto the CMC-agar medium, MC-agar medium and GC-agar medium via point inoculation. They were cultured for 48 h at 28°C, and then dyed with 5 mL 1 mg/mL Congo red for 10 min, followed by decolourisation with 1mol/L NaCl solution for 5 min. The bacteria with transparent hydrolytic ring represented cellulolytic bacteria. Next, the top 5 bacteria with better cellulose degradation capacity were screened based on the speed of the transparent hydrolytic ring and the size of the bacterial colony. The diameter of the hydrolytic ring surrounding the colony (D) and the colony diameter (d) on the plate were measured with a microscope. Each strain was measured 3 times and the D/d ratio was used as the index for preliminary determination of the degradation ability. A statistical analysis of D/d values was performed by using SPSS 20.0 software.

MOLECULAR BIOLOGICAL IDENTIFICATION OF STRAINS

The colonies were cultivated in TSB at 28°C for 24 h, and harvested by centrifugation at 4000 g for 10 min. DNA was extracted using a bacterial genomic DNA extraction kit (Tsingke Biotechnology Co., Ltd , Beijing, China). The 16S rRNA gene sequence was amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACG ACTT-3') according to the method described by Weisburg et al.[18] Following the completion of the PCR reaction, the PCR products were detected via 1% agarose gel electrophoresis (AGE). The amplified DNA segments were sent to Tsingke Biotechnology Co., Ltd for DNA sequencing. Later, BLAST comparative analysis of sequences was carried out in the NCBI database (blast.ncbi.nlm.nih.gov) to determine the species relationships. The phylogenetic tree was constructed using the neighbor-joining (NJ) technique in MEGA7.0 software.

RESULTS

ISOLATION AND SCREENING OF INTESTINAL CELLULOLYTIC BACTERIA IN RED SWAMP CRAYFISH

A total of 12 single colonies on TSA plate were selected for analysis. They were separated and purified repeatedly on three cellulose plates. After treatment with Congo red, five strains produced hydrolytic rings. As shown in **Table 1**, the 5 cellulolytic bacterial strains were E, M, G, C and H on the CMC-agar medium ranging in size from big to small, E, C, M, G and H on the microcrystalline cellulose plate (**Table 2**), as well as H, G, C, M and E on the cellobiose plate (**Table 3**). The strain E presented the strongest degradation of cellulose in both CMC-agar medium and MC-agar medium. Although strain H had a weak ability to decompose cellulose directly, it exhibited the strongest capacity for the decomposition of cellobiose.

Strain C was a small and smooth yellowish white round colony. It contains an obvious bulge in the center, with smooth and wet edges. Strain E was a yellow oval colony, with obvious boundaries between the center and edges. The edges were extremely thin and it was thick in the center, with a rough surface. Strain G was a milk-white oval colony, with smooth and wet surfaces. It carried a yellowish and swollen center. Strain H was a light red large round colony, without obvious boundaries between the center and edges. The center was slightly thick and white, while the edges were light red. The surface was smooth and wet. Strain C was a light yellow round colony with moderate size and mild boundary sense at the edges. The external edges were white and the central bulge was yellow.

IDENTIFICATION OF STRAINS AND CONSTRUCTION OF THE PHYLOGENETIC TREE

The 16S rDNA gene of the screened strains was amplified by PCR to obtain nearly 1500 bp. The 16S rDNA sequences (about 1500 bp) of the five screened cellulolytic bacteria were submitted to GenBank (**Table 4**). Similarity search was performed on NCBI database. The 16S rDNA sequence sim-

Table 1. The comparison of diameter of colonies, hydrolyzing zones of cellulose-degrading bacteria colonies on CMC-agar medium

Medium	Strain number	Diameter of colonies (d/mm)	Hydrolyzing zones (D/mm)	D/d
CMC-agar	C	1.27±0.71	4.00±0.00	3.75±1.77
	E	1.33±0.58	5.33±0.58	4.50±1.80
	G	1.67±0.58	6.00±1.00	3.83±1.04
	H	1.13±0.75	3.67±3.78	2.76±1.29
	M	0.77±0.21	3.00±1.00	4.01±1.48

Table 2. The comparison of diameter of colonies, hydrolyzing zones of cellulose-degrading bacteria colonies on MC-agar medium

Medium	Strain number	Diameter of colonies (d/mm)	Hydrolyzing zones (D/mm)	D/d
MC-agar	C	2.00±0.00	3.00±0.00	1.50±0.00
	E	2.00±0.01	3.33±0.58	1.67±0.29
	G	4.33±0.58	5.33±0.58	1.23±0.03
	H	5.67±0.58	6.5±0.71	1.19±0.02
	M	3.33±0.58	4.5±0.71	1.29±0.06

Table 3. The comparison of diameter of colonies, hydrolyzing zones of cellulose-degrading bacteria colonies on GC-agar medium

Medium	Strain number	Diameter of colonies (d/mm)	Hydrolyzing zones (D/mm)	D/d
GC-agar	C	6.00±0.00	8.33±1.53	1.39±0.26
	E	3.33±0.58	3.67±0.58	1.11±0.19
	G	5.00±0.00	8.33±1.53	1.67±0.31
	H	4.67±0.58	9.67±0.58	2.08±0.14
	M	8.67±0.58	12.00±1.73	1.38±0.11

ilarity between strain C and *Citrobacter sp*. reached 99.86%. The 16S rDNA sequence similarity between strain E and *Staphylococcus sp*. was 100%. The 16S rDNA sequence similarity between G and *Acinetobacter johnsonii* reached 99.71%.The 16S rDNA sequence similarity between strain H and *Shewanella* sp. was 99.79%. The 16S rDNA sequence similarity between strain M and *Aeromonas caviae* was 99.79%. The phylogenetic tree (**Figure1**) showed that strains H and M had a close genetic relationship and strain C was clustered independently, indicating that strain C had a distant genetic relationship with other four strains.

DISCUSSION

Five cellulolytic bacteria were screened from the intestinal tract of *P. clarkii* via pure culture method in this experiment. According to the results of 16S rRNA molecular identification, the strains C, E, G, H, and M were *Citrobacter sp*., *Staphylococcus sp*., *Acinetobacter johnsonii*, *Shewanella sp*., and *Aeromonas caviae*, respectively. Feng et al.[17] screened cellulolytic bacteria from the intestinal tracts of *P. clarkii* using CMC-agar medium, and were identified as *B. velezensis* and *B. subtilis*. The screened bacteria differed from those detected in this experiment, which might be attributed to differences in intestinal flora caused by different living environments.[19]

Deng et al.[20] explored the intestinal flora of *P. clarkii* cultured in ponds via high-throughput sequencing and found that the dominant bacteria included predominantly *Citrobacter sp., Hafnia enterococcus, Candidatus Bacilloplasma, Bacteroides sp., Clostridiaceae unclassified genus,* and *Shewanella*. Feng et al.[21] studied the intestinal flora of *P. clarkii* which were cultured in-door by high-throughput sequencing and found that the dominant bacteria were *Candidatus Bacilloplasma, Bacteroides, Acinetobacter, Dysgonomonas, Tyzzerella3, Aeromonas,* and *Shewanella*. Li et al.[14] found that *Aeromonas, Enterobacter, Enterococcus, Citrobacter sp.* and *Bacillus* were the major cellulolytic bacteria in the intestines of grass carp. In this experiment, *Citrobacter sp*. and *Shewanella sp*. were the dominant culturable bacteria in the intestinal tract of *P. clarkii* and they play an important role in cellulose degradation of *P. clarkii*.

Table 4. Representative sequences of cultured cellulolytic bacteria isolated from the gut of *P. clarkia

Strain number	Description	Coverage	Similarity	Accession no
C	Citrobacter sp. 519C4 16S ribosomal RNA gene, partial sequence	100.00%	99.86%	KT764985.1
E	Staphylococcus sp. strain NAS4 16S ribosomal RNA gene, partial sequence	100.00%	100.00%	MN519627.1
G	Acinetobacter johnsonii strain cqsV17 16S ribosomal RNA gene, partial sequence	100.00%	99.71%	MN826586.1
H	Shewanella sp. MR-4, complete genome	100.00%	99.79%	CP000446.1
M	Aeromonas caviae strain ATCC 15468 16S ribosomal RNA, partial sequence	99.00%	99.79%	NR029252.1

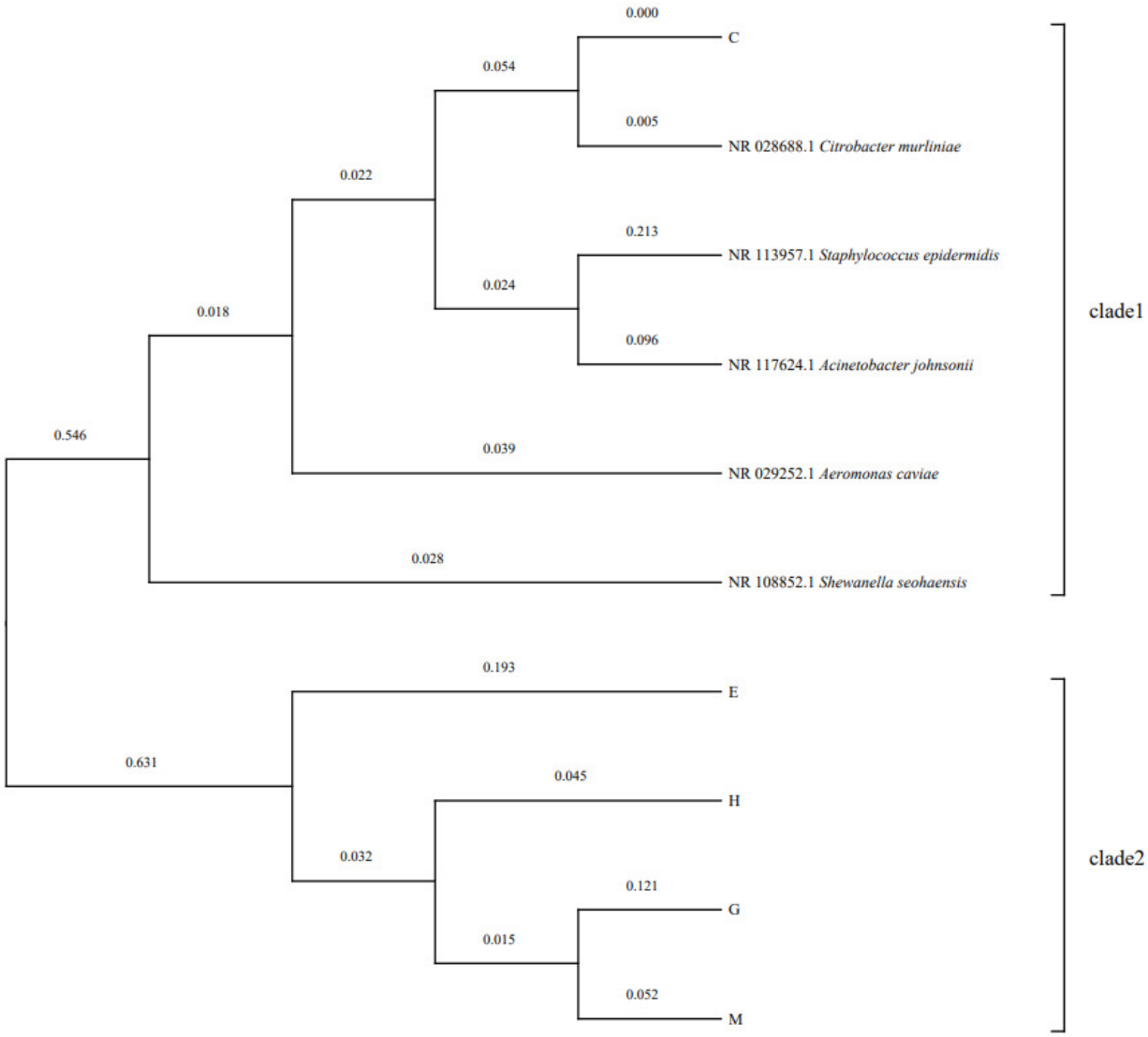

Figure 1. Phylogenetic tree based on 16S rDNA cloning sequences of intestinal cellulolytic bacteria based on *P. clarkia

Huang[22] found that *Citrobacter sp.* was the cellulolytic bacteria in the intestinal tract of *Holotrichia parallela* larvae, which was consistent with the results of the present study. He[23] reported that *Citrobacter sp.* was a common nonpathogenic bacterium in animal intestinal bacteria. Deng et al.[24] also found that *Citrobacter sp.* was a normal core flora in the intestinal tracts of *P. clarkii*. However, Chen et al.[25] found that *C. freundii* was pathogenic in *Micropterus salmoides*. Liu et al.[26] also found that *C. freundii* was a pathogenic bacterium associated with tail-rot disease of *P. clarkii*. Hence, the pathogenicity of *Citrobacter sp.* identified in this study will be further studied.

Aeromonas caviae is a pathogenic bacterial species infecting humans. It may adversely affect bile duct or urinary

system and exhibits strong pathogenicity against *Litopenaeus vannamei* and *Macrobrachium rosenbergii*.[27-29] *Aeromonas caviae* as cellulolytic bacterial species in animal intestines has yet to be reported. The pathogenicity of *Aeromonas caviae* in *P. clarkii* requires further investigation.

Staphylococcus acts as a lignocellulolytic bacterium infecting white ants.[30] It is a cellulolytic bacterium infecting the intestinal tracts of *Ornithophora auriculata* larvae,[31] which is consistent with our experimental findings. Staphylococci are a group of gram-positive cocci. *S. aureus* is one of the most important pathogenic bacteria, which cause global food poisoning. The screened staphylococcus in this experiment exhibits a completely different colonial morphology compared with *S. aureus*. Whether the screened *Staphylococcus* can be used as a probiotic for *P. clarkii* has yet to be investigated.

Acinetobacter johnsonii acted as a cellulolytic bacterial species in *Coptotermes formosanus*[32] and degraded lignocellulose in the soils and straw compost.[33] *Acinetobacter johnsonii* generally is not a pathogenic bacterium. Studies reported no human infection caused by *Acinetobacter johnsonii*.[34] *Acinetobacter johnsonii* was not pathogenic in the intestinal tracts of grass carp.[35] Hence, it has great potential for research and development as a probiotic.

Shewanella sp. was cellulolytic in the intestinal tract of *P. clarkii* in the present study. Shi[36] also demonstrated that *Shewanella sp*. was cellulolytic in the intestinal tract of grass carp. *Shewanella sp*. is the dominant core intestinal bacteria in grass carp, and it can be used as a probiotic in aquatic animals. After feeding grass carp with *Shewanella sp*., the immunity was enhanced and the levels of total protein (TP), albumin, globulin and complement C3 level in serum were increased, accompanied by significant upregulation of IL-8, IL-1β, TNF-α and lysozyme-C.[37] Hence, *Shewanella sp*. has great potential for use as a probiotic.

In summary, *Aeromonas caviae* is pathogenic to the human body and aquatic animals, and it cannot be used as a probiotic in *P. clarkii*. *Citrobacter sp.* and *Staphylococcus sp.* will be further evaluated and utilized for further research and development of probiotics. *Acenatobacter johnsonii* and *Shewanella sp*. have great potential for application as probiotics in forage for *P. clarkia,* suggesting the need for further studies. The present study can provide a theoretical basis for the development of probiotics in the feed of *P. clarkia.*

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AUTHORS' CONTRIBUTION

Conceptualization: Liye Shao (Equal), Pinhong Yang (Equal). Methodology: Liye Shao (Equal), Ronghua Wang (Equal). Data curation: Liye Shao (Equal), Xiangyan Qiu (Equal), Jiaxun Li (Equal), Junming Chen (Equal), Ronghua Wang (Equal). Investigation: Liye Shao (Equal), Ronghua Wang (Equal). Formal Analysis: Liye Shao (Equal), Xiangyan Qiu (Equal), Ronghua Wang (Equal). Writing – original draft: Liye Shao (Equal), Xiangyan Qiu (Equal). Writing – review & editing: Ronghua Wang (Equal), Pinhong Yang (Equal). Resources: Ronghua Wang (Equal). Funding acquisition: Pinhong Yang (Equal). Supervision: Pinhong Yang (Lead).

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