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Characterization and identification of virulent *Klebsiella oxytoca* isolated from abalone (*Haliotis diversicolor supertexta*) postlarvae with mass mortality in Fujian, China

Junpeng Cai [a,b,*], Zexiu Wang [a], Chuanhua Cai [c], Yiping Zhou [c]

[a] *College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, China*
[b] *College of Biosciences and Bioengineering, South China University of Technology, Guangzhou 510640, China*
[c] *Institute of South China Oceanography, Chinese Academy of Sciences, Guangzhou 510310, China*

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Abstract

An epidemic of mass mortality of abalone (*Haliotis diversicolor supertexta*) postlarvae aged 40 days or less has existed across south coast of China since the second half of 2002. Among 20 bacterial strains isolated from diseased abalone postlarvae on 2216E marine agar plates during an outbreak of postlarval disease in August 2005, a predominant strain (designated strain 20) was demonstrated to be virulent to postlarvae with an LD_{50} value of 1.0×10^5 colony forming units (CFU ml^{-1}) on day 4, while the other 19 strains were either avirulent (16 strains) or weakly virulent (3 strains). The same bacterium could be re-isolated from postlarvae after bacterial challenge using 2216E marine agar plates. Preliminary toxicity tests of ECPs of strain 20 revealed that at 2.77 mg protein ml^{-1}, crude ECPs completely liquefied postlarvae within 24 h, leaving only shells. API 20E analysis identified strain 20 as *Klebsiella oxytoca*. 16S and ITS rDNA sequencing and phylogenetic analyses further confirmed this identification. Antibiotic susceptibility tests showed that strain 20 exhibited 94% of susceptibility to 16 various antibiotics tested and only showed resistance to streptomycin. Results of this work demonstrated that *K. oxytoca* is also linked to this epidemic in Fujian, China. This is considered to be the first report regarding *K. oxytoca* involved in the mass mortality of postlarval abalone in south China and the world.

Keywords: Abalone *Haliotis diversicolor supertexta*; Postlarvae; Mass mortality; *Klebsiella oxytoca*; Virulence tests; ECPs; API 20E; 16S–ITS rDNA sequencing

1. Introduction

Abalone are highly prized gastropod mollusc, especially in Eastern Asia. Among them, *Haliotis diversicolor supertexta*, is a commercially important species cultured on the south coast of China, including Taiwan (Chen, 1989). However, since the second half of 2002, an epidemic of postlarval abalone mass mortality has swept across southern China. This occurred when postlarvae were less than 40 days post fertilization and were less than 2.5 mm in shell length. Diseased postlarvae showed gross signs of lethargy, a whitened body colour when viewed in natural light. Muscle bodies were shrunken, resulting in larger shells than the bodies. Most diseased postlarvae fell-off biofilms in the end while some others, thought still on the films, were left with empty shells. The occurrence of postlarval abalone disease reduced this small abalone production from 10 million yuan annually to about 2 million yuan, and left this budding industry in a dire situation.

Although upon examination of the cause(s) of this epidemic, Cai et al. (2006a, b, c) have revealed that *Shewanella algae*, *Vibrio parahaemolyticus* and *V. alginolyticus* are

* Corresponding author. Address: College of Light Industry and Food Sciences, Department of Food Sciences, South China University of Technology, Wushan Road, TianHe District, Guangzhou, Guangdong province 510640, China.
E-mail address: febjpcai@scut.edu.cn (J. Cai).

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pathogens that are associated with mass mortality of postlarval abalone in Shanwei area, it is still not known if these pathogens are also responsible for the other outbreaks across southern China. To help answer this question, in this study, we aimed to examine an outbreak of postlarval abalone disease occurred in Fujian province, China and identify the causative agent responsible for the outbreak.

2. Materials and methods

2.1. Abalone postlarvae sampling and bacterial isolation

Abalone postlarvae used for bacterial isolation were from an abalone farm in Zhanpu of Fujian province, China. They were collected from diseased postlarvae during an outbreak of postlarval disease in August 2005, in which postlarval mortality was between 80% and 90%, with some 50 postlarvae left on any given one of the biofilms (normally, there should be ca. 500–600 postlarvae on a film of a size of 80 cm length × 80 cm width). As the diseased postlarvae were tiny (ca. 0.4 mm of shell length on average), to maximize the chances of bacterial isolation, 10 diseased (whitened) postlarvae of 18 days post fertilization were collected with a pipette fitted with a sterile 1-ml tip from diatom films in a nursery pond that was experiencing a disease outbreak and placed in a sterile polystyrene Petri dish containing 0.5 ml sterile phosphate-buffered saline (PBS: 0.8775% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 10% glycerol, pH 7.2). They were transferred to the microbiological laboratory for bacterial analysis on the farm. After rinsing thoroughly with sterile PBS, all 10 postlarvae were pooled together as one sample and homogenized in a sterile glass grinder with 0.5 ml sterile PBS. A 10-fold dilution series ranging from 10^{-1} to 10^{-5} was prepared from this homogenate. To obtain bacteria rather than just vibrios, the broad range growth medium, viz. Zobell's 2216E Marine medium, was chosen. Each sample in the dilution series was plated out on 2216E marine (Difco, supplemented with 2.5% NaCl) agar plates using 0.1 ml inoculum per plate. All dilutions were plated in triplicate. Plates were then incubated at 25 °C. After 5–7 days incubation, morphologically different bacterial colonies were aseptically picked and purified by repeatedly streaking onto fresh agar plates. After this, purified colonies were multiplied in 2216E broth and stored in glycerol (10%) at −70 °C.

2.2. Virulence tests

Virulence tests for all bacterial isolates were carried out as reported by Cai et al. (2006b). Briefly, apparently healthy 20-day-old postlarvae together with their attached diatom films were first cut and made bacteria-free by bathing in mixed antibiotics solution (chloramphenicol, 20 mg L^{-1}; norfloxacin, 10 mg L^{-1}; erythromycin, 15 mg L^{-1}; gentamicin, 40 mg L^{-1}; penicillin G, 200,000 IU L^{-1}; and polymyxin B, 300 mg L^{-1}). Then bacteria-free postlarvae were held in 2-L beakers supplied with 1 L of autoclaved, aerated 3% salinity sand-filtered seawater at 25 °C, pH 8. The LD_{50} tests, with batches of 20 abalone postlarvae per dose, were conducted by addition of serial bacterial suspensions (24 h bacterial culture, 10^3–10^6 CFU ml^{-1}, final concentrations) into the beakers (Trevors and Lusty, 1985). Sterile PBS was added into other beakers as parallel controls. Each bacterial dose in the tests, including the control, had 3 beakers as a group to form a triplicate experiment.

Mortalities were recorded daily for 4 days post addition of bacterial suspensions. Re-isolation and identification of the bacteria from moribund postlarvae after virulence tests were conducted with 2216E marine agar plates. LD_{50} values were calculated by the method of Reed and Muench (1938). LD_{50} of $>10^8$ CFU ml^{-1} is considered avirulent whist that of 10^4–10^5 CFU ml^{-1} is considered virulent according to the criteria set by Mittal et al. (1980).

2.3. Bacterial characterization and identification

In this study, characterization and identification were performed only with the dominant strain, i.e. strain 20.

Strain 20 was characterized and identified using standard morphological, physiological and biochemical plate and tube tests and API 20E system (ATB system, BIOMÉRIEUX SA, Marcy-I'Etoile, France). API 20E strips were incubated for 18–24 h at 32 °C. The reactions were compared with the reference strain of *Klebsiella oxytoca* ATCC 13182^T (ATCC, American Type Culture Collection, Rockville, MD, USA).

To characterize strain 20 at the molecular level, 16S and ITS rDNA sequencing and phylogenetic analyses were also performed.

Strain 20 was grown overnight at 30 °C in 2216E broth (Difco) with shaking at 150 rpm. Cells were harvested by centrifugation at 5000 rpm for 30 min at 4 °C and washed and re-suspended with sterile 1× TE buffer (10 mM Tris–HCl, 100 mM EDTA, pH 8.0). Genomic DNA extraction and 16S–ITS PCR amplification were performed as stated by Cai et al. (2006b), using primers 63F (5′-cag gcc taa cac atg caa gtc-3′, positions 63–83 of 16S, *Escherichia coli* numbering) and 4R [5′-tga ctg cca a(g)gg cat cca-3′, positions 40–21 of 23S, *E. coli* numbering]. PCR products were purified using a PCR purification kit (Takara, China) according to the manufacturer's instructions after the confirmation of successful amplifications by electrophoresis of 5 μl PCR products on a 1% agarose gel. PCR direct sequencing was done as reported by Thompson et al. (1992). Nucleotide sequence data was deposited in GenBank Nucleotide Sequence Data Libraries. Computer program Blastn was employed to identify the species closest to the sequence of strain 20. Phylogenetic trees were constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath and Sokal, 1973) based on 16S rDNA and ITS sequences separately and their