Isolation, identification and phylogenetic analysis of a pathogen of *Haliotis diversicolor supertexta (L.)* with mass mortalities

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Abstract

Studies were conducted to determine a disease outbreak in 14 day old post-larvae of abalone (*Haliotis diversicolor supertexta*) which caused mass mortality in July 2010 in Shanwei, China. Twenty-nine bacterial strains were isolated from a sample pool of 10 diseased post-larval abalones on 2216E marine agar plates during a natural outbreak of the disease. Among them, a dominant isolate (referred to as strain 21) was found to be highly virulent to post-larvae in experimental challenge tests, with an LD_{50} value of 1.0×10^4 colony forming units (CFU) mL^{-1} on day 3. API 20NE kits and 16S rDNA sequence analysis, identified strain 21 as *Oceanomonas doudoroffii*. It was susceptible to 10 and moderately susceptible to 1 of the 16 antibiotics examined when antibiotic sensitivities of the bacterium were assayed. Results of this study implicated *Oceanomonas doudoroffii* strain 21 as a cause of mortalities in post-larval abalone from Shanwei, China.

Keywords: *Haliotis diversicolor supertexta*, Massive death, Challenge test, *Oceanomonas doudoroffii*, 16S rDNA sequencing

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Introduction

Production of various species of the genus *Haliotis* is a very important part of aquaculture in the Orient since they have been regarded as very precious seafood. However, from 2002 onwards, mass mortalities of post-larvae of the small abalone, *Haliotis diversicolor supertexta L.*, have persisted and forced many abalone farms to close (Cai et al., 2006a) Diseased post-larval abalone became pale and lethargic with atrophied muscles. Most of the post-larvae came off the diatom bio-films on which they grew one to two days after these symptoms appeared. Some did not come off the bio-films but their shells were left empty.

Upon examination of the cause(s) of these outbreaks, Cai et al. (2006a,b, c), revealed that *Vibrio parahaemolyticus*, *Shewanella algae*, *V. alginolyticus* and *Klebsiella oxytoca* were all associated with mass mortality of post-larval abalone in Shanwei and Fujian. However, it is not yet known if any of these pathogens were also responsible for other outbreaks in different farms in the same region. To this end, we report the isolation and characterization of *Oceanomonas doudoroffii* as a cause of mass mortalities in the post-larval abalone, *Haliotis diversicolor supertexta (L.)* in July 2010 in Shanwei, China.

Materials and methods

Bacterial isolation

The whitened (diseased) post-larval abalone, that used for the bacterial isolation were from an abalone farm in Shanwei of Guangdong Province, China. They were collected during an outbreak of post-larval disease in July 2010 when the post-larvae were just 14 days post fertilization. Post-larvae were on average approximately 0.3mm in length. To maximize chances of bacterial isolation, ten were collected with a pipette fitted with a sterile 1-mL tip from bio-films, and placed in a sterile polystyrene petri dish containing 0.5 mL sterile phosphate buffered saline (PBS) of 0.01 mol/L(pH 7.2). Then they were transferred to the on site microbiological laboratory and homogenized in a sterile glass grinder with 0.5 mL sterile PBS after being rinsed 3 times with PBS. A 10-fold dilution series ranging from 10^{-1} to 10^{-5} was prepared from this homogenate. 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. After 4-6 days incubation at 25 °C, representative colonies, according to their different morphologies and abundance on the culture media, were selected and purified for characterization and identification.

Abalone and virulence test

Abalone of 20 days post-fertilization with an average shell length of ca. 0.9 mm were collected from an abalone farm in Shenzhen, Guangdong Province. There was no incidence of disease outbreaks at this site during the hatching season. These abalone were used for experimental challenge tests which were run according to the protocol described by Cai, Chen, Thompson and Li (2006a). Briefly, each post-larval abalone, with its attached

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diatom film, was first cut into pieces of approximately 1 cm^2 or less and placed in a sterile 2-L beaker and rinsed three times with 500 mL autoclaved and aerated seawater containing six antibiotics (Takara, China)(chloramphenicol, 20 mg/ L; norfloxacin, 10 mg/L; erythromycin, 15 mg/L; gentamicin, 40 mg/L; penicillin G, 200,000 IU/L; and polymyxin B, 300 mg /L) and then immersed in 1 L of the same mixed antibiotic solution for a day with change of the water every 6 h. Once pour plate technique had confirmed bacteria-free status, challenge tests were carried out. A series of dilutions of four different bacterial suspensions (24 h bacterial culture, 10^3–10^6 CFU/mL, final concentrations) was run in the tests with 2-L beakers as containers (Trevors and Lusty, 1985). To each 2-L beaker, 1L of autoclaved, aerated 3% salinity sand-filtered seawater, 20 bacteria-free post-larval abalones and the appropriate concentration of bacteria were added. Three beakers without any added bacteria were used as negative controls.

All twenty-nine isolates, recovered from the diseased abalone homogenate, were subjected to challenge tests, performed over a three-day period and in triplicate. LD_{50} values were calculated on day 3 according to Reed & Muench(1938). LD_{50} values of $>10^8$ CFU mL^{-1} were considered a-virulent, and values of between 10^4 and 10^5 CFU/mL were considered virulent, while values between 10^6 and 10^7 CFU mL^{-1} were considered weakly virulent in line with the virulence criteria of Mittal et al.(1980).

To verify Koch's postulate in the case of the most virulent strain, each of the post-larvae in the challenge tests was picked-up under a magnifier, thoroughly rinsed with sterile PBS and used for re-isolation and identification of bacteria. Mortality was attributed to the bacterium isolated if it was recovered in pure culture from dead post-larvae (Brock et al., 1994).

Bacterial characterization

The pure cultures recovered from the sample pool of 10 diseased post-larval abalone were checked to stablish if they could grow on thiosulfate citrate bile salt sucrose medium (TCBS; Difco, Detroid, USA) supplemented with 2.5 % (w/v) NaCl. Further experiments were carried out only to characterize the most virulent bacterial strains identified in the challenge tests.

The most abundant and virulent bacterial strain (viz. strain 21) was subjected to standard morphological, physiological, and biochemical examination. Gram-reaction, oxidase, catalase and hydrolysis of aesculin and gelatin were tested as described by Baumann et al. (1972) and Smibert and Krieg (1994). The ability to use phenol as the sole carbon source was determined on minimal media that contained 2% NaCl (w/v) and 4 mM phenol for up to 7 days incubation. Leifson's oxidation–fermentation medium for marine bacteria (Leifson, 1963) was used to test acid production from carbohydrates with 1% (w/v) of each compound. Other biochemical tests were carried out using API 20NE test kits (ATB system, BIOMÉRIEUX SA, Marcy-I'Etoile,

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France) according to the manufacturer's instructions.

In order to characterize strain 21 at the molecular level, 16S and its rDNA sequencing and phylogenetic analyses was performed. Strain 21 was grown overnight at 25 °C in marine broth with shaking at 180 rpm. Cells from cultures were harvested by centrifugation at 10,000 g for 2 min, rinsed, and then suspended in 1x TE buffer (10 mM Tris-HCl, 100 mM EDTA, pH 8.0). Genomic DNA extraction and PCR amplification of 16S rDNA of strain 21 were run as reported by Cai et al. (2006a). After confirmation of successful amplifications by electrophoresis of 5 µL PCR products on a 1% agarose gel, the products were purified using a PCR purification kit (Takara, China). PCR direct sequencing was done as reported by Thompson et al. (1992). Sequence data was then deposited in GenBank. The computer program Blast was used to identify the species which was phylogenetically closest to the sequence of strain 21 and an evolutionary tree was determined using the Neighbor-Joining method (Saitou and Nei, 1987) with the program EGA version 4.0. The stability of inter-relationships of bacteria in the phylogenetic tree was assessed by performing bootstrap analysis with 500 re-samplings.

Sensitivity of strain 21 to 16 various chemotherapeutic agents

To investigate whether antibiotics could be used in the control of strain 21, sensitivity experiments were performed as reported by Cai et al. (2006b).The discs used in the assay included three cell wall synthesis inhibitors (ampicillin, 10 µg; cefamezin, 30 µg; and penicillin G, 10 IU), one cell membrane permeability interferer (polymyxin B, 300 µg), eight protein synthesis inhibitors (amikacin, 30 µg; chloramphenicol, 30 µg; erythromycin, 15 µg; gentamicin, 10 µg; kanamycin, 30 µg; neomycin, 30 µg; streptomycin, 10 µg; and tetracycline, 30 µg) and four nucleic acid synthesis inhibitors (ciprofloxacin, 5 µg; norfloxacin, 10 µg; novobiocin, 5 µg; and trimethoprim-sulfamethoxazole, 25 µg). *Escherichia coli* ATCC 25922 was also included in the analysis as a control bacterium. Antibiotic sensitivity to a particular antibiotic (i.e. sensitive, intermediately sensitive or resistant) was assessed according to the recommended cut-off levels of the zone size.

Results

Bacterial isolation

Twenty-nine representative colonies were isolated from sampled diseased post-larval abalone during the disease outbreak in July 2010 in Shanwei, Guangdong Province. Selection of these colonies was based on differences in morphology and their relative abundances on marine agar plates. One isolate, designated as strain 21, was found to be the dominant colony on the plates.

Bacterial challenge tests

Bacterial challenge tests were performed on all 29 strains. Results showed that strain 21 was the most virulent isolate, killing 100 % of the post-larvae on day 3, and had a LD_{50} value of 1.0×10^{4} CFU mL^{-1} (Table 1) while 3 of the other isolates (strains 1, 9 and 29) were weakly virulent

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with LD_{50} values ranging from 2.3 $\times 10^6$ CFU mL^{-1} to 4.5 $\times 10^7$ CFU mL^{-1}, and the remaining 25 isolates were classified as a-virulent with LD_{50} values greater than 1.0 $\times 10^8$ CFU mL^{-1}. No mortality was observed in the controls. Gross symptoms observed in moribund post-larvae during natural outbreaks were also observed in the post-larvae of the challenge tests.

Bacteriological examinations showed that strain 21 was re-isolated as pure cultures from moribund post-larvae from challenge tests and thus Koch's postulates were fulfilled.

Table 1: LD_{50} (50 % Lethal Dose) values on day 3 post-infection, calculations based on challenge tests carried out on the 29 isolates recovered from diseased post-larval abalone

Strain	LD_{50} value* (CFU mL^{-1})	Strain	LD_{50} value (CFU mL^{-1})
1	5.5×10^6	16	2.5×10^9
2	3.2×10^8	17	4.1×10^8
3	4.0×10^8	18	3.1×10^8
4	1.4×10^9	19	3.2×10^9
5	1.5×10^8	20	1.5×10^8
6	1.7×10^8	21	1.0×10^4
7	2.8×10^8	22	1.3×10^8
8	1.5×10^8	23	1.5×10^8
9	2.3×10^6	24	3.6×10^8
10	1.6×10^9	25	1.8×10^8
11	4.5×10^8	26	1.7×10^8
12	4.1×10^8	27	6.0×10^9
13	2.1×10^8	28	2.9×10^8
14	1.5×10^8	29	4.5×10^7
15	1.8×10^9		

Bacterial characterization

Both standard tests and API 20NE analysis (Table 2) showed that strain 21 was a Gram-negative rod and an obligate aerobic. It was positive for oxidase, catalase, Na^+ requirement for growth, nitrate reduction, caprate, malate and citrate utilization. It was negative for indole production, glucose acidification, arginine dihydrolase, gelatin and aesculin hydrolysis and utilization of glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, and adipate. Growth was detected when cultivated in minimal salts medium that contained 2% NaCl and phenol as the sole carbon and energy source. Based on these features and comparisons with those of the reference strain DSM 7028^T (=ATCC 27123^T) in API 20NE tests, strain 21 conformed to the description of *Oceanomonas doudoroffii* (Geoffrey et al., 2001).

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Table2: Physiological characteristics of stain 21 as revealed by API 20NE test

Character	Strain 21	DSM 7028^T
Reduction of nitrate to nitrite	+	+
Indole production	-	-
Acidification of glucose	-	-
Arginine hydrolase present	-	-
Urease present	-	-
Gelatin hydrolysis	-	-
Aesculin hydrolysis	-	-
β-Galactosidase present	-	-
Utilization of:		
Glucose	-	-
Arabinose	-	
Mannose	-	-
Mannitol	-	-
N-Acetylgcosamine	-	-
Maltose	-	-
Gluconate	-	-
Caprate	+	+
Adipate	-	-
Malate	+	+
Citrate	+	+
Phenylacetate	-	-
Requirement for Na^+	+	+

To further characterize strain 21 at the molecular level, 16S rDNA PCR sequencing analysis was performed. PCR amplification of 16S rDNA and the its region yielded an amplicon of approximately 1.8 kp in size. PCR direct sequencing was carried out and sequences of 16S rDNA and its region of strain 21 were submitted to GenBank under accession No. **DQ026025**. The 16S rRNA gene sequence (1331 bases) of strain 21 was manually aligned using the clustal method in the megalign program (DNAStar) with its related bacteria in the Order Aeromonadales(Martin and Joseph, 2004). A similarity search done by using the BLAST program showed that strain 21 had the closest relationship with *Oceanomonas doudoroffii* DSM 7028^T (99% similarity, AB019390) (Fig. 1) and displayed 96.9% and 95% sequence similarities with *O. baumannii* strain GB6 (AF168367) and *Oceanisphaera litoralis* DSM 15406 (AJ550470), respectively. Thompson et al.(2004) pointed out that 16S sequence similarities of ≥ 97% could be considered as the same species, and thus strain 21 was identified at the molecular level as *Oceanomonas doudoroffii*.

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Table 3: Sensitivity of strain 21 to various chemotherapeutic agents

Chemotherapeutic agents	Disc content (μg)	Sensitivity [a]
Penicillin G	10 IU	MS
Cefamezin	30	S
Kanamycin	30	S
Gentamicin	10	S
Chloramphenicol	30	S
Polymyxin B	300	S
Norfloxacin	10	R
Ampicillin	10	S
Streptomycin	10	S
Amikacin	30	R
Trimethoprim-sulfamethoxazole	25	S
Erythromycin	15	S
Tetracycline	30	R
Ciprofloxacin	5	S
Novobiocin	5	R
Neomycin	30	R

[a] R: resistance; S: sensitive; MS: moderately sensitive

Sensitivity to chemotherapeutic agents

Strain 21 was susceptible to 10 and moderately susceptible to 1 of the 16 chemotherapeutic agents examined, as shown in Table 3, indicating that strain 21 exhibited 62.5 % susceptibility to the antibiotics examined and was resistant to norfloxacin, amikacin, tetracycline, novobiocin and neomycin.

Discussion

In the current study, even though there were 29 representative colonies isolated on 2216E agar plates from the diseased post-larval abalone sampled during the disease outbreak in July 2010 in Shanwei, Guangdong Province, only strain 21 was predominant on the agar plates. Live bacterial challenge tests also revealed that only strain 21 was highly virulent to post-larvae as judged by the criteria of Mittal et al. (1980).

Among the other isolates, 3 were weakly virulent and the rest 25 were a-virulent. This suggests that post-larval abalone is more susceptible to strain 21 than its counterparts (LD_{50} values are between 2.3×10^6 and 1.5×10^9). And it further supports the notion that "The abundance of one dominant type of bacterium in recently dead abalone implicated this bacterium in their mortality", as stated by Nicolas et al. (2002).

Strain 21 was Gram-negative rods, obligate aerobic, oxidase-positive and could utilize malate, succinate, citrate and galactose for growth. On the basis of these biochemical characteristics (Table 2) and comparison with the API 20E results of the reference strain DSM 7028^T (=ATCC 27123^T), strain 21 was confirmed to be *Oceanomonas doudoroffii*. The results of the 16S rDNA sequencing revealed that strain 21 shared 99% similarity with the type strain of *O. doudoroffii* viz. strain Bry (ATCC 27123^T, accession number AB019390). Strain 21 may therefore be considered as *O. doudoroffii*. Phylogenetic

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analysis based on the nearly complete 16S rRNA gene sequence of strain 21 indicated that *O. doudoroffii* ATCC 27123T and *O. baumannii* ATCC 700832T(Geoffrey et al., 2001)were its closest phylogenetic neighbors. *Oceanisphaera litoralis* DSM 15406T(Ivanova et al., 2005), *Tolumonas auensis* DSM 9187T(Fischer et al., 1996) and the genus *Aeromonas* were more distantly related. This relationship is displayed in the 16S rRNA gene sequence dendrogram based on the additive treeing algorithm of DeSoete (1983)(Fig. 1).

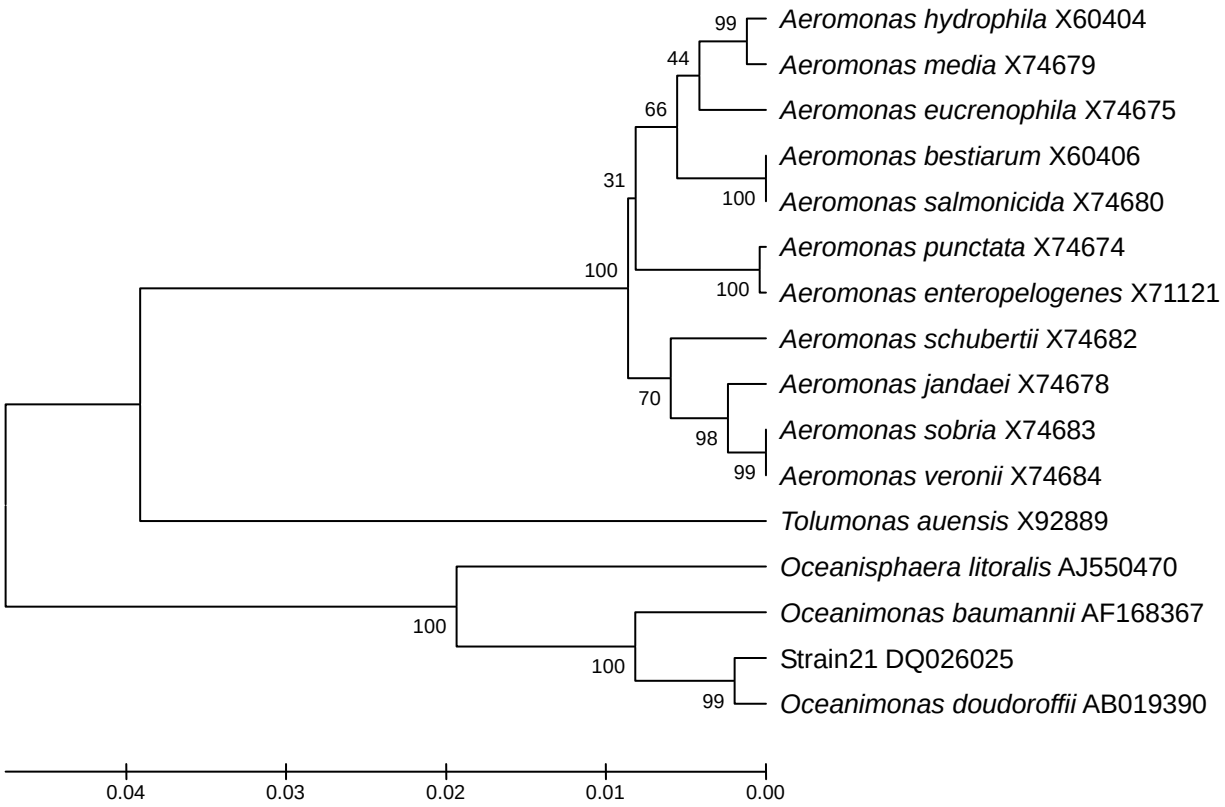

Fig. 1 Dendrogram of 16S rRNA gene sequence relatedness, showing that strain 21 is phylogenetically closely related to members of the genus *Oceanimonas*, and shares 99% 16S rRNA gene sequence similarity with *Oceanomonas doudoroffii* DSM 7028T. Numbers at branching points refer to bootstrap values (500 re-samplings).

From the live bacterial challenge experiments, a dose of 1 $\times 10^6$ CFU/mL of strain 21 was able to cause 100 % mortalities within 3 days, and had an LD_{50} value as low as 1.0 $\times 10^4$ CFU/mL, while the other 3 strains were classified as weak virulent with LD_{50} values ranging from 1.0 $\times 10^6$ CFU mL^{-1} to 1.0 $\times 10^7$ CFU mL^{-1}, and the remaining 25 isolates were classified as a-virulent with LD_{50} values greater than 1.0 $\times 10^8$ CFU mL^{-1}. This is well correlated with the severity of the actual outbreak of post-larval disease as only about 90% of the mortality occurred in that particular outbreak and about 50 post-larvae were still retained on nearly every bio-film after the administrations of antibiotics.

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Furthermore, the same bacterium could be re-isolated from moribund/whitened post-larvae after bacterial challenge and similar gross signs (turning white and falling off diatom films) in the post-larvae of abalone *Haliotis diversicolor supertexta* as that observed in natural outbreaks.

The threat of *Oceanomonas doudoroffii* is mainly to post-larval stages and thus prevention of infection by *O. doudoroffii* is essential to eliminate this threat. Strain 21 was susceptible to 10 and moderately susceptible to 1 of the 16 chemotherapeutic agents examined, as shown in Table 3. This result suggests the possibility of employing some appropriate chemotherapeutic agents in disease control. However, it must be pointed out that some of the antibiotics tested, including chloramphenicol, are prohibited for use in aquaculture, so for the long term, environmental friendly strategies are required to the prevent disease outbreaks on abalone farms. A possible alternative to the use of antibiotics may be the application of probiotics, especially since Macey and Coyne (2005) demonstrated improved growth and increased disease resistance in *Haliotis midae*, after treating the culture system with probiotics.

Ongoing studies are focused on the proteomics of these extracellular products as targets for therapy or prophylaxis of this deadly infection in post-larvae of abalone *Haliotis diversicolor supertexta*.

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