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Studies on bacterial pathogens isolated from diseased torafugu (*Takifugu rubripes*) cultured in marine industrial recirculation aquaculture system in Shandong Province, China

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Abstract

In spring of 2011, an epidemic outbreak of torafugu with high mortality occurred in an aquafarm with marine industrial recirculation aquaculture system (MIRAS) in Yantai, Shandong Province, China. The diseased fish showed anorexia, haemorrhaging and festering fin and skin and swelling internal organs. Forty-five dominant bacterial isolates were obtained from the diseased fish, and were found to belong to 12 species according to 16S rRNA gene sequences. One strain from each species was selected to test the pathogenicity, and five strains were showed to be virulent to zebrafish. Whereas *Enterovibrio nigricans* Fr42 was highly virulent with the LD_{50} of 7.8×10^4 CFU g^{-1}, *Photobacterium swingsii* Fr23, *Vibrio owensii* Fr40, *V. harveyi* Fr51 and *V. rotiferianus* Fr71 were moderately virulent with the LD_{50} of 1.7×10^6 to 8.4×10^6 CFU g^{-1}. Both the bacteria and their extracellular products of the five strains were found to show phospholipase, caseinase, gelatinase, amylase and/or lipase activities. The production of *N*-acyl homoserine lactones (AHLs) of the five strains was detected by three different AHLs biosensors, and three of them were found to produce AHLs by at least one kind of biosensor. This is the first study describing various opportunistic bacterial pathogens of fish cultured in MIRAS in China.

Keywords: Fish disease, Torafugu, Bacterial pathogen, AHLs, MIRAS

Introduction

Over the past 30 years, aquaculture of China has soared from small-scale commerce to farming at a large-scale capacity, accounting for about 6.3% of world aquatic products in 1978 to more than 80% in 2010 (Wang 2009; Oehlenschlager 2010). Being the largest fishery producer in the world, China plays crucial role in improving the aquaculture industry worldwide.

Torafugu (*Takifugu rubripes*, a pufferfish; Temminck et Schlegel, 1850) is one of the most prestigious edible fish species in many Asian countries and it occupies a particular place in the diet culture in China, Korea and Japan. Because of its tasty and nourishing flesh (Gao, Huang, Xia, Lu & Liu 2011) and high market price (Kikuchi, Furuta, Iwata, Onuki & Noguchi 2009), torafugu has become one of the most extensive and economically important maricultured fish on the north coast of China, such as Shandong and Liaoning Provinces.

However, due to the nonscientific husbandry, environmental deterioration and other reasons, diseases were bloomed and a variety of pathogenic microorganisms were isolated from torafugu and other fish, and some of them are devastating, such as *Vibrio* (Qin, Zhang, Chen, Fang & Xu 2008; Wang, Yu, Hu, Li, Liu & Jiang 2008; Mohi, Kuratani, Miyazaki & Yoshida 2010), *Myxidium* (Tun, Yokoyama, Ogawa & Wakabayashi 2000), *Listonella* (Zhang, Qin, Yan, Xu, Bi & Qin 2009b), *Photobacterium* (Wang, Han, Li, Chen & Zhang 2007) and *Iridovirus* (Miyata, Matsuno, Jung, Danayadol & Miyazaki 1997).

Marine industrial recirculation aquaculture system (MIRAS), designed by and used in the aquafarm, is mariculture systems in which about 95% of total water volume can be reused through

continual treatment, including removing fine solids and dissolved organic materials by foam fractionation and filtration, killing bacteria by ultraviolet irradiation and ozone, regulating temperature, adding oxygen and degassing carbon dioxide, etc. (Hutchinson, Jeffrey, O'Sullivan, Casement & Clarke 2004; Liu 2011; Martinsa, Eding, Verdegem, Heinsbroek, Schneider, Blancheton, Roque d'Orbcastel & Verreth 2012). Although the disease control treatment existing in MIRAS, but it still cannot guarantee that the cultured fish are free of bacteriosis.

From April to June in 2011, a paroxysmal epidemic outbreak with a high mortality occurred in several aquafarms in Shandong Province, China. To investigate the causative agent contributed to the epidemic outbreak of fish, bacterial pathogens were isolated from diseased torafugu cultured in MIRAS. Five pathogenic bacterial strains were selected for further analysis. For the first time *Vibrio* spp., *Enterovibrio nigricans* and *Photobacterium swingsii* have been clearly demonstrated to be involved in the bacteriosis of torafugu cultured in MIRAS.

Materials and methods

Isolation of bacterial pathogens

In spring of 2011, a paroxysmal epidemic outbreak of torafugu with a high mortality occurred in an aquafarm with MIRAS in Yantai, Shandong Province. Diseased fish were washed three times with sterile (121°C, 20 min) 0.85% (w/v) saline solution (SNS). The samples of ulcerative skin and fin, liver, ascites, gall bladder, kidney, spleen and intestine were excised from living fish and homogenized in 1 mL SNS with aseptic technique, and were inoculated immediately onto TSN (TSA + 2.5% sodium chloride), marine agar 2216 and TCBS (Difco) plates. After 24–48 hours incubation at 28°C, prominent colonies in plates which contained dense more or less pure culture growth were picked up according to morphological characteristics and abundance on the culture media and streaked onto fresh media for three times to obtain pure cultures. All the strains were preserved at −80°C in SNS supplemented with 15% (v/v) glycerol.

DNA extraction and 16S rRNA gene amplification

Each of the dominant isolate was inoculated into 2216E agar slants and incubated at 28°C for 24 h. The genomic DNA was extracted and purified using standard methods (Sambrook 1989; Ausubel, Brent, Kingston, Moore, Seidman, Smith & Struhl 1995). Two sets of universal primers B8F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and B1510: 5′-GGTTACCTTGTTACGACTT-3′ synthesized by BioSune Biotechnology (Shanghai) were used to amplify the 16S rRNA genes of the isolates. The PCR reaction system and the reaction conditions were according to Jin, Wang, Yu, Yan and Zhang (2010). The PCR products (~1500 bp) were analysed by 1% agarose gel electrophoresis and sequenced by Beijing Genomics Institute (BGI, Shenzhen, China).

Phylogenetic analysis based on 16S rRNA gene sequence

The obtained sequences were aligned and compared with other bacterial 16S rRNA sequences available in GenBank of NCBI database and in EzTaxon server 2.1. According to the results of challenge tests (as following), five representative strains were selected for further analysis. A phylogenetic tree of these five bacteria was constructed by the Neighbour-Joining method using the MEGA 5.05 software, and bootstrap analysis with 1000 replicates was adopted to estimate the relative branch support of the tree (Ke, Lu, Ye, Gao, Zhu & Huang 2012).

Challenge tests

Overnight bacterial cultures at 28°C on 2216E were used to prepare suspensions in SNS. The viable cell concentration was established by preparing serial 10-fold dilutions of the bacterial suspension in fresh SNS to 10^4 cells mL^{-1} assessed by observing optical density at 600 nm (OD_{600}), and spreading 0.1 mL volumes of each dilution over the surface of duplicate plates of 2216E with incubation at 28°C for 2 days.

Zebrafish (*Danio rerio*) (average weight = 0.3–0.4 g) was used as model to evaluate the pathogenicity of representative isolates following the methods described before (Wang *et al.* 2007; Kinkel, Eames, Philipson & Prince 2010). Zebrafish was reared statically at room temperature (18 ± 2°C) in 50 L tanks filled with filtered (0.45 μm) freshwater for 7 days to confirm their health status before use, and half volume of rearing water was changed daily. Groups of 10 zebrafish, four groups per representative isolate, were infected by intraperitoneal

injection with 20 μL of serial dilutions of bacterial suspensions. Controls were injected with 20 μL SNS. The infected zebrafish were maintained for 3 weeks, and disease signs and mortalities were recorded. The lethal dose 50% (LD_{50}) values were calculated using the probit method described by Wardlaw (1985). The re-isolation and identification of bacteria from moribund zebrafish were carried out to confirm the pathogens.

AHLs production test

The production of AHLs of the five representative isolates obtained from diseased torafugu was tested using three different biosensors. The plate assays of *Chromobacterium violaceum* CV026 (Throup, Bainton, Bycroft, Williams & Stewart 1995) and *Agrobacterium tumefaciens* A136 (Fuqua & Winans 1996) were carried out according to Ravn, Christensen, Molin, Givskov and Gram (2001), and the bioassay of *Escherichia coli* JB523 (Andersen, Heydorn, Hentzer, Eberl, Geisenberger, Christensen, Molin & Givskov 2001) was carried out according to Yang, Kim, Park, Lee, Park, Song, Joo, Kim, Hahn and Kim (2009).

Analysis of virulence-related factors

Assays for detecting virulence-related factors of the bacterial isolates and their ECP were carried out according to previous protocols (Liu, Lee & Chen 1996; Austin, Austin, Sutherland, Thompson & Swings 2005; Natrah, Ruwandeepika, Pawar, Karunasagar, Sorgeloos, Bossier & Defoirdt 2011) with minor modifications. For bacterial isolates, a colony of each bacterial strain was picked onto phospholipase, caseinase, gelatinase, amylase, lipase and blood plates by sterile toothpicks. ECP was extracted as directed by Abbass, Sharifuzzaman and Austin (2010), and 200 μL ECP was transferred to Oxford Cups plated on test agar under sterile conditions. All assays were carried out in triplicate.

Results

Characterization of diseased fish and bacterial isolation

The major symptoms of diseased torafugu collected from the aquafarm were haemorrhaging, festering fin and skin and swelling entrails (such as liver, gall bladder, kidney, or intestine) (Fig. 1). A total of 45 dominant bacterial strains with distinguishable morphological characteristics were isolated from different tissues of diseased torafugu.

Bacterial identification by 16S rRNA gene sequencing and phylogenetic analysis

All the 45 isolates from diseased torafugu were identified by means of 16S rRNA gene sequence analysis. These isolates belong to 12 species, including

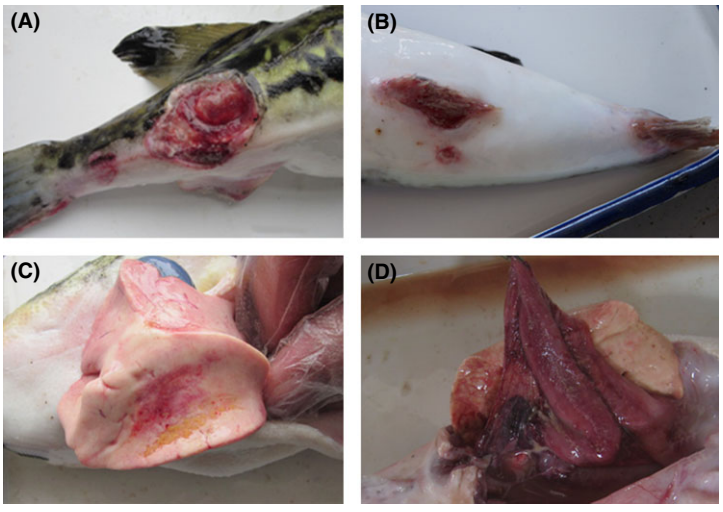

Fig. 1 Photograph of skin ulceration disease of torafugu. Arrows indicated the area of pathological changes. (A) and (B) torafugu suffering from skin and fin ulceration; (C) hyperaemic liver of diseased torafugu; (D) swelling and congestive intestine of diseased torafugu.

Vibrio harveyi (27 isolates), *V. scophthalmi* (1 isolates), *V. rotiferianus* (3 isolates), *V. campbellii* (2 isolates), *V. owensii* (1 isolate), *Enterovibrio nigricans* (1 isolate), *Bacillus methylotrophicus* (3 isolates), *B. amyloliquefaciens* (3 isolates), *B. safensis* (2 isolates), *Photobacterium swingsii* (1 isolate), *Pseudoalteromonas tetraodonis* (1 isolate) and *Alteromonas macleodii* (1 isolate). Almost 50% of them were isolated from ulceration of fin and skin, among which 82% were *Vibrio* species. *Vibrio* species were also isolated from swelling entrails. For instance, *V. harveyi* was isolated from spleen (3 isolates), kidney (3 isolates), liver (2 isolates), gall bladder (1 isolate), and intestine (1 isolate). Moreover, *V. scophthalmi* (1 isolate) was isolated from gall bladder, while one strain *V. campbellii* was isolated from liver and spleen respectively.

The phylogenetic tree (Fig. 2) was constructed using the 16S rRNA gene sequences of the five representative isolates and their closely related species, and showed that the five bacterial isolates clustered into three clades. While *V. owensii* Fr40, *V. harveyi* Fr51 and *V. rotiferianus* Fr71 clustered into the vibrio clade, *E. nigricans* Fr42 clustered in a second clade and *P. swingsii* Fr23 clustered into a third clade. *P. swingsii* Fr23 and *V. rotiferianus* Fr71 were isolated from ascites of diseased torafugu, *V. owensii* Fr40 and *V. harveyi* Fr51 were from ulceration of fin, and *E. nigricans* Fr42 were from swelling and/or hyperaemia of intestine.

Challenge tests

One strain from each of the 10 species was selected to test the pathogenicity by challenge test, and five strains were found to be virulent to zebrafish. LD_{50} values of *P. swingsii* Fr23, *V. owensii* Fr40, *E. nigricans* Fr42, *V. harveyi* Fr51 and *V. rotiferianus* Fr71 were 8.4×10^6, 3.1×10^6, 7.8×10^4, 2.7×10^6 and 1.7×10^6 CFU g^{-1} body weight within 2 weeks respectively. All mortalities of the bacterial challenge tests occurred within 5 days. The zebrafish of control group injected with SNS were alive and normal till the end of the experiment.

The diseased zebrafish showed symptoms similar to some extent of the original disease; swelling and hyperaemia of entrails were the most common clinical signs. *Enterovibrio nigricans* Fr42 showed high virulence, whereas *P. swingsii* Fr23, *V. owensii* Fr40, *V. harveyi* Fr51 and *V. rotiferianus* Fr71 were moderately virulent.

AHLs production tests

The expression of virulence factors of many fish pathogens in aquaculture were found to be controlled by quorum sensing (QS) (Swift *et al.* 1997; Henke & Bassler 2004; Defoirdt, Bossier, Sorgeloos & Verstraete 2005; Han, Li, Qi, Zhang & Bossier 2010). Therefore, the AHLs (QS signal molecule) production of five representative isolates were detected by three different biosensors *C. violaceum* CV026, *A. tumefaciens* A136 and *E. coli* JB523. Each biosensor responds to a different range of AHLs. While *C. violaceum* CV026 responds mainly to short-chained unsubstituted AHLs which induce a purple pigment formation in the bioassay medium (McClean, Winson, Fish, Taylor, Chhabra, Camara, Daykin, Lamb, Swift, Bycroft, Stewart & Williams 1997), *A. tumefaciens* A136 is positive for most 3-oxo-*N*-AHLs molecules with blue colouration (Shaw, Ping, Daly, Cha, Cronan, Rinehart & Farrand 1997) in the bioassay medium due

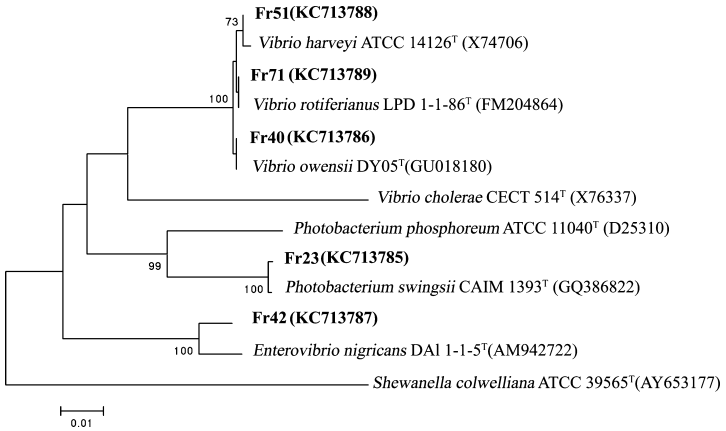

Fig. 2 Phylogenetic tree of the bacterial isolates constructed using Neighbour-Joining method in Mega 5.05.

to active expression of the lacZ reporter gene. *E. coli* JB523 detects a broad spectrum of AHLs molecules by giving rise to green fluorescence (Andersen *et al.* 2001). Among them, *V. owensii* Fr40, *E. nigricans* Fr42 and *V. harveyi* Fr51 were detected to produce AHLs by A136 and/or JB523. However, *P. swingsii* Fr23 and *V. rotiferianus* Fr71 were not detected to produce AHLs by all the three biosensors (Table 1). None of the isolates were detected to produce AHLs by CV026 except the positive control.

Analysis of virulence-related factors

Although the mechanism of pathogenesis in bacterial pathogens is not completely understood, a few virulence-related factors, such as extracellular products with proteolytic or haemolytic activity, have been identified in *Vibrio* (Zhang & Austin 2000; Frans, Michiels, Bossier, Willems, Lievens & Rediers 2011; Darshanee Ruwandeepika, Sanjeewa Prasad Jayaweera, Paban Bhowmick, Karunasagar, Bossier & Defoirdt 2012), *Edwardsiella* (He & Zhang 2009) and *Photobacterium* (Romalde 2002). Hence, both the isolates and their ECPs were detected for phospholipase, caseinase, gelatinase, amylase, lipase activities and haemolysis (Table 2). *Vibrio owensii* Fr40, *V. harveyi* Fr51 and *V. rotiferianus* Fr71 and their ECPs were found to produce all those virulence-related factors. However, some virulence-related factors were positive for the whole cells but negative for ECPs. For example, *E. nigricans* Fr42 was positive for caseinase, gelatinase and lipase, while its ECP was negative for lipase.

Discussion

Recently, it was reported that global aquaculture has supplied 78% more fish, three times more crustaceans and 60% more molluscs for human in the last two decades when aquaculture of China has been developing at full speed (Peng 2013). The aquaculture in China has been the fastest growing food-producing sector and the aquatic product output has ranked first in the world since 1989 (Wang 2009; Bostock, McAndrew, Richards, Jauncey, Telfer, Lorenzen, Little, Ross, Handisyde, Gatward & Corner 2010).

Presently, however, commercial production of fish in China is hampered by variable mortalities in fish farms from different regions. Although the pathogenesis of diseases in mariculture has not been stated clearly, bacteria have been considered the major pathogen in most of the reported fish diseases. Various pathogens have been proved to be involved in torafugu diseases since 1990s, such as *Edwardsiella tarda* (Ma & Wang 1994), *Vibrio* spp. and *Flexibacter* spp. (Zhang 2002; Wang *et al.* 2008) and *Streptococcus* spp. (Du 2003). However, the pathogens mentioned above were not well clarified, and the characteristics of suspected pathogens were not described except *V. harveyi* (Wang *et al.* 2008), *V. penaeicida* (Qin *et al.* 2008) and *V. ichthyoenteri* (Zhang, Chen, Yan, Fang, Qin & Xu 2009a). Moreover, when torafugu disease broke out, rearing conditions (such as salinity, temperature and pH) and the pathogenic bacterial species involved in the disease were rather complex and different from place to place. So, no certain disease or its pathogen(s) in torafugu has been confirmed so far. Haemorrhaging and festering fin and skin, swelling entrails and other similar

Table 1 AHLs production of the bacterial pathogens using three different biosensors

	Biosensors		
	CV026	A136	JB523
Photobacterium swingsii Fr23	–	–	–
Vibrio owensii Fr40	–	–	+
Enterovibrio nigricans Fr42	–	+	+
Vibrio harveyi Fr51	–	–	+
Vibrio rotiferianus Fr71	–	–	–

+, positive reaction; –, negative reaction.

Table 2 The results of virulence-related factor analysis of the bacterial pathogens (a) and ECP (b)

	Fr23	Fr40	Fr42	Fr51	Fr71
(a) Enzyme activities of the isolates					
Phospholipase	+	+	–	+	+
Caseinase	–	+	+	+	+
Gelatinase	+	+	+	+	+
Amylase	+	+	–	+	+
Lipase	+	+	+	+	+
Haemolysin	+	+	–	+	+
(b) Enzyme activities of the ECP					
Phospholipase	+	+	–	+	+
Caseinase	–	+	+	+	+
Gelatinase	+	+	–	+	+
Amylase	+	+	–	+	+
Lipase	+	+	+	+	+
Haemolysin	–	+	–	+	+

+, positive reaction; –, negative reaction.

symptoms might be common in many diseases caused by different pathogens in which *Vibrio was* frequently observed. It suggests that the bacteria involved in the disease might be opportunistic, but not specific.

The results of 16S rRNA gene sequence analysis demonstrated that around 73% of the dominant isolates belonged to *Vibrio* spp., among which nearly 80% were *V. harveyi*. Challenge tests indicated that five strains were virulent to zebrafish namely *P. swingsii* Fr23, *V. owensii* Fr40, *E. nigricans* Fr42, *V. harveyi* Fr51 and *V. rotiferianus* Fr71. Notably, *Photobacterium*, *Enterovibrio* and *Vibrio* all belong to the family of Vibrionaceae (2004). The results based on the virulence-related factor analysis showed that both the isolates and ECP of *V. owensii* Fr40, *V. harveyi* Fr51 and *V. rotiferianus* Fr71 were positive for phospholipase, caseinase, gelatinase, amylase, lipase and haemolysis, and these three isolates were proved to be moderately virulent by challenge tests using zebrafish as model fish. However, live cells displayed more enzymatic activities than the ECP, for example, in *E. nigricans* Fr42. It was thought that some cell envelope-associated proteinases were related with many of these activities (Zhang & Austin 2000). Alternatively, it is conceivable that some enzymatic substrates might be catalysed by enzymes within live cells. Wang *et al.* (2008) also isolated *V. harveyi* from diseased torafugu with ulceration of skin, and found that ECP of *V. harveyi* had amylase and casease activities, which was speculated to be one of the reasons resulting in partial tissue damage (Wang, Yu, Yuan & Jiang 2010). In our study, nonetheless, *E. nigricans* Fr42 showed high virulence while it was negative for phospholipase, amylase and haemolysin, and its ECP was only positive for caseinase and lipase. It may be results from the differences between different pathogens. The structure and virulence of the virulence-related factors in the same category, such as haemolysin, varied in different isolates even in the same species (Zhang & Austin 2005). Moreover, perhaps there is unknown pathogenic mechanism in *E. nigricans* Fr42, which needs to be further studied.

QS in Gram-negative bacteria refers to the ability of a bacterium to sense information, generally using *N*-AHLs as signal molecules, from other cells in the population when they reach a critical concentration (i.e., a Quorum) and communicate with them (Deep, Chaudhary & Gupta 2011). It is thought that pathogenic bacteria utilize QS as part of their pathogenic lifestyle, with regard to a mechanism to minimize host immune responses by delaying the production of tissue-damaging virulence factors until sufficient bacteria have amassed and are prepared to overwhelm host defence mechanisms and establish infection (Kievit & Iglewski 2000; Deep *et al.* 2011). Many fish and shrimp pathogens in aquaculture, such as *Vibrio*, *Aeromonas* and *Edwardsiella*, were reported to control virulence factor expression by QS system (Swift *et al.* 1997; Henke & Bassler 2004; Defoirdt *et al.* 2005; Han *et al.* 2010). Consequently, QS-disrupting techniques, including inhibition of synthesis of AHLs and binding to corresponding receptor and inactivation of AHLs, have emerged recently and are regarded as one of the most promising alternatives to antibiotics for the control of bacterial disease in aquaculture (Defoirdt, Sorgeloos & Bossier 2011). AHLs production tests showed that *V. owensii* Fr40, *E. nigricans* Fr42 and *V. harveyi* Fr51 could produce AHLs by A136 and/or JB523. More remarkably, only *E. nigricans* Fr42, showing the highest virulence in this study, was found to produce AHLs by both A136 and JB523. Compared with *V. owensii* Fr40 and *V. harveyi* Fr51, *E. nigricans* Fr42 was negative for phospholipase, amylase and haemolysin. Natrah *et al.* (2011) reported that QS in *V. harveyi* positively regulate caseinase and gelatinase activity, negatively regulate phospholipase activity whereas haemolysin and lipase were found to be independent of QS. The relation between AHLs production and virulence-related factors in *V. owensii* Fr40, *E. nigricans* Fr42 and *V. harveyi* Fr51 await the outcome of further research. However, our results showed that some isolates positive for AHLs were avirulent to zebrafish (data not shown). One appropriate reason for this phenomenon is that QS controls many physiological process besides regulating virulence-related factor expression, such as bioluminescence (Bassler, Wright, Showalter & Silverman 1993) and conjugation (Miller & Bassler 2001), so positive for AHLs of a pathogen is not always equivalent to high pathogenicity.

The environment in MIRAS was quite different from that in common pond or cage farming in many aspects, such as organic content (remnant feed and faeces), nutrient element content, etc. (Liu 2011), which probably led to different microbial community structure. In this study, for the first time five different bacterial pathogens were

obtained from diseased torafugu cultured in MIRAS. *Vibrio* spp. are also common in diseased torafugu cultured in net cages or ponds, such as *V. harveyi* (Wang *et al.* 2008; Mohi *et al.* 2010), *V. penaeicida* (Qin *et al.* 2008) and *V. ichthyoenteri* (Zhang *et al.* 2009a). However, *P. swingsii* and *E. nigricans* have not been reported to be involved in diseased torafugu so far. Moreover, the pathogenic mechanism of mixed isolates infection remains unclear and await the outcome of further research.

In conclusion, *E. nigricans*, *V. harveyi*, *V. owensii* and *P. swingsii* have been clearly demonstrated to be involved in the bacteriosis of torafugu cultured in MIRAS for the first time. Although their precise pathogenic mechanisms remain still unclear and needs to be further studied, to some extent QS and extracelluar enzymes might contribute to their pathogenicity.

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References

Abbass A., Sharifuzzaman S.M. & Austin B. (2010) Cellular components of probiotics control *Yersinia ruckeri* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **33**, 31–37.

Andersen J.B., Heydorn A., Hentzer M., Eberl L., Geisenberger O., Christensen B.B., Molin S. & Givskov M. (2001) gfp-Based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Applied and Environmental Microbiology* **67**, 575–585.

Austin B., Austin D., Sutherland R., Thompson F. & Swings J. (2005) Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia nauplii*. *Enviromental Microbiology* **7**, 1488–1495.

Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A. & Struhl K. (1995) *Short Protocols in Molecular Biology* (3rd edn). Wiley, New York, 870 pp.

Bassler B.L., Wright M., Showalter R.E. & Silverman M.R. (1993) Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Molecular Microbiology* **9**, 773–786.

Bostock J., McAndrew B., Richards R., Jauncey K., Telfer T., Lorenzen K., Little D., Ross L., Handisyde N., Gatward I. & Corner R. (2010) Aquaculture: global status and trends. *Philosophical Transactions of the Royal Society B* **365**, 2897–2912.

Darshanee Ruwandeepika H.A., Sanjeewa Prasad Jayaweera T., Paban Bhowmick P., Karunasagar I., Bossier P. & Defoirdt T. (2012) Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the *Harveyi* clade. *Reviews in Aquaculture* **4**, 59–74.

Deep A., Chaudhary U. & Gupta V. (2011) Quorum sensing and bacterial pathogenicity: from molecules to disease. *Journal of Laboratory Physicians* **3**, 4–11.

Defoirdt T., Bossier P., Sorgeloos P. & Verstraete W. (2005) The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. *Environmental Microbiology* **7**, 1239–1247.

Defoirdt T., Sorgeloos P. & Bossier P. (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology* **14**, 1–8.

Du J.Y. (2003) Stretococcusis of tiger puffer (*Fugu rubripes*). *Hebei Fisheries* **1**, 30–31 (In Chinese).

Frans I., Michiels C.W., Bossier P., Willems K.A., Lievens B. & Rediers H. (2011) *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *Journal of Fish Diseases* **34**, 643–661.

Fuqua C. & Winans S.C. (1996) Conserved cis-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. *Journal of Bacteriology* **178**, 435–440.

Gao L.J., Huang Y.Q., Xia L.J., Lu J.X. & Liu S.C. (2011) Comparison of flesh quality of farmed fugu, *Takifugu rubripes* from different culture models. *Journal of Fisheries of China* **35**, 1668–1670 (In Chinese).

Han Y., Li X., Qi Z., Zhang X.-H. & Bossier P. (2010) Detection of different quorum-sensing signal molecules in a virulent *Edwardsiella tarda*strain LTB-4. *Journal of Applied Microbiology* **108**, 139–147.

He Y. & Zhang X.-H. (2009) Advances in virulence-related factors of *Edwardsiella tarda*. *Periodical of Ocean University of China* **5**, 979–987 (In Chinese).

Henke J.M. & Bassler B.L. (2004) Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *Journal of Bacteriology* **186**, 6902–6914.

Hutchinson W., Jeffrey M., O'Sullivan D., Casement D. & Clarke S. (2004) Recirculating aquaculture systems: minimum standards for design, construction and management. Prepared for the Inland Aquaculture Association of South Australia Inc. 1–70.

Jin G., Wang S.S., Yu M., Yan S.L. & Zhang X.-H. (2010) Identification of a marine antagonistic strain JG1 and establishment of a polymerase chain reaction detection technique based on the *gyrB* gene. *Aquaculture Research* **41**, 1867–1874.

Ke X.L., Lu M.X., Ye X., Gao F.Y., Zhu H.P. & Huang Z.H. (2012) Recovery and pathogenicity analysis of Aerococcus viridans isolated from tilapia (*Orecohromis niloticus*) cultured in southwest of China. *Aquaculture* **342–343**, 18–23.

Kievit T.R.D. & Iglewski B.H. (2000) Bacterial quorum sensing in pathogenic relationships. *Infection and immunity* **68**, 4839–4849.

Kikuchi K., Furuta T., Iwata N., Onuki K. & Noguchi T. (2009) Effect of dietary lipid levels on the growth, feed utilization, body composition and blood characteristics of tiger puffer *Takifugu rubripes*. *Aquaculture* **298**, 111–117.

Kinkel M.D., Eames S.C., Philipson L.H. & Prince V.E. (2010) Intraperitoneal injection into adult zebrafish. JoVE. 42. Available at: http://www.jove.com/details.php?id=2126 (accessed 8 August 2011). doi: 10.3791/2126

Liu Y. (2011) Research progress on marine industrial recirculating aquaculture technology. *Journal of Agricultural Science and Technology* **13**, 50–53.

Liu P.C., Lee K.-K. & Chen S.-N. (1996) Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn *Penaeus monodon*. *Letter Applied Microbiology* **22**, 413–416.

Ma D.Y. & Wang X.C. (1994) Common disease of *Fugu rubripes* in culture. *Marine Sciences* **1994**, 16–17.

Martinsa C.I.M., Eding E.H., Verdegem M.C.J., Heinsbroek L.T.N., Schneider O., Blancheton J.P., Roque d'Orbcastel E. & Verreth J.A.J. (2012) New developments in recirculating aquaculture systems in Europe: a perspective on environmental sustainability. *Aquacultural Engineering* **43**, 83–93.

McClean K.H., Winson M.K., Fish L., Taylor A., Chhabra S.R., Camara M., Daykin M., Lamb J.H., Swift S., Bycroft B.W., Stewart G.S.A.B. & Williams P. (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acyl homoserine lactones. *Microbiology* **143**, 3703–3711.

Miller M.B. & Bassler B.L. (2001) Quorum sensing in bacteria. *Annual Review Microbiology* **55**, 165–199.

Miyata M., Matsuno K., Jung S.J., Danayadol Y. & Miyazaki T. (1997) Genetic similarity of *Iridoviruses* from Japan and Thailand. *Journal of Fish Diseases* **20**, 127–134.

Mohi M.M., Kuratani M., Miyazaki T. & Yoshida T. (2010) Histopathological studies on *Vibrio harveyi*-infected tiger puffer, *Takifugu rubripes* (Temminck et Schlegel), cultured in Japan. *Journal of Fish Diseases* **33**, 833–840.

Natrah F.M.I., Ruwandeepika H.A.D., Pawar S., Karunasagar I., Sorgeloos P., Bossier P. & Defoirdt T. (2011) Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. *Veterinary Microbiology* **154**, 124–129.

Oehlenschlager J. (2010) Fish products throughout the world-source, quality and safety. *Ernahrungs Umschau* **57**, 490–496.

Peng X.X. (2013) Proteomics and its applications to aquaculture in China: infection, immunity, and interaction of aquaculture hosts with pathogens. *Developmental and Comparative Immunology* **39**, 63–71.

Qin G.M., Zhang X.J., Chen C.Z., Fang H.Y.B.L. & Xu J. (2008) Biological characterization of *Vibrio penaeicida* sp. nov. from *Takifugu rubripes* L. *Oceanologia Et Limnologia Sinica* **39**, 228–233 (In Chinese).

Ravn L., Christensen A.B., Molin S., Givskov M. & Gram L. (2001) Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHLs-production kinetics. *Journal of Microbiological Methods* **44**, 239–251.

Romalde J.L. (2002) Photobacterium damselae subsp. piscicida: an integrated view of a bacterial fish pathogen. *International Microbiology* **5**, 3–9.

Sambrook J., Fritsch E.F. & Maniatis T.M. (eds.) (1989) *Molecular Cloning: A Laboratory Manual* (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 350 pp.

Shaw P.D., Ping G., Daly S.L., Cha C., Cronan J.E., Rinehart K.L. & Farrand S.K. (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proceedings of the National Academy of Sciences USA* **94**, 6036–6041.

Swift S., Karlyshev A.V., Fish L., Durant E.L., Winson M.K., Chhabra S.R., Williams P., Macintyre S. & Stewart G.S. (1997) Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. *Journal of Bacteriology* **179**, 5271–5281.

Throup J.P., Bainton N.J., Bycroft B.W., Williams P. & Stewart G.S.A.B. (1995) Signaling in bacteria beyond bioluminescence. In: *Bioluminescence and Chemiluminescence: Fundamental and Applied Aspects* (ed. by A.K. Cambell, L.J. Kricka, & P.E. Stanley), pp 89–92. Wiley, Chichester, UK.

Tun T., Yokoyama H., Ogawa K. & Wakabayashi H. (2000) Myxosporeans and their hyperparasitic microsporeans in the intestine of emaciated tiger puffer. *Fish Pathology* **35**, 145–156.

Wang W. (2009) Current situation and development trend of aquaculture in China. *Fishery Guide to be Rich* **7**, 12–18 (In Chinese).

Wang Y., Han Y., Li Y., Chen J.X. & Zhang X.-H. (2007) Isolation of *Photobacterium damselae* subsp. *piscicida* from diseased tongue sole (*Cynoglossus semilaevis* Gunther) in China. *Acta Microbiologica Sinica* **47**, 763–768.

Wang B., Yu L.P., Hu L., Li Y., Liu S.F. & Jiang Z.Q. (2008) Isolation and identification of bacteriosis pathogen from cultured *Fugu rubripes* with canker of skin. *Journal of Fishery Sciences of China* **15**, 352–358 (In Chinese).

Wang B., Yu L.P., Yuan T. & Jiang Z.Q. (2010) Pathogenicity of extracellular products of *Vibrio harveyi* to *Fugu obscurus*. *Journal of Fishery Sciences of China* **17**, 88–96 (In Chinese).

Wardlaw A.C. (1985) *Practical Statistics for Experimental Biologists*. John Wiley and Sons, Chichester.

Yang Y.H., Kim T.W., Park S.H., Lee K., Park H.Y., Song E., Joo H.S., Kim Y.G., Hahn J.S. & Kim B.G. (2009) Cell-free *Escherichia coli*-based system to screen for quorum-sensing molecules interacting with quorum receptor proteins of *Streptomyces coelicolor*. *Appllied and Environmental Microbiology* **75**, 6367–6372.

Zhang W.G. (2002) Common diseases of *Fugu rubripes* and the prevention. *Fishery Guide to be Rich* **11**, 34 (In Chinese).

Zhang X.-H. & Austin B. (2000) Pathogenicity of *Vibrio harveyi* to salmonids. *Journal of Fish Diseases* **23**, 93–102.

Zhang X.-H. & Austin B. (2005) Haemolysins in *Vibrio* species. *Journal of Applied Microbiology* **98**, 1011–1019.

Zhang X.J., Chen C.Z., Yan B.L., Fang H., Qin G.M. & Xu J. (2009a) Studies on biological characteristics of pathogenic *Vibrio ichthyoenteri* isolated from pufferfish (*Takifugu rubrips* L.). *Acta Oceanologica Sinica* **33**, 1118–1125 (In Chinese).

Zhang X.J., Qin G.M., Yan B.L., Xu J., Bi K.R. & Qin L. (2009b) Phenotypic and molecular characterization of pathogenic *Listonella anguillarum* isolated from half-smooth tongue sole *Cynoglossus semilaevis*. *Acta Oceanologica Sinica* **31**, 112–122 (In Chinese).

