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Isolation and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. from nearshore marine sediments

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Summary

A total of 94 actinomycete strains were isolated from the marine sediments of a shrimp farm, 87.2% belonged to the genus *Streptomyces*, others were *Micromonospora* spp. Fifty-one percent of the actinomycete strains showed activity against the pathogenic *Vibrio* spp. strains. Thirty-eight percent of marine *Streptomyces* strains produced siderophores on chrome azurol S (CAS) agar plates. Seven strains of *Streptomyces* were found to produce siderophores and to inhibit the growth of *Vibrio* spp. *in vitro*. Two of them belonged to the Cinerogriseus group, the most frequently isolated group of *Streptomyces*. The results showed that streptomycetes could be a promising source for biocontrol agents in aquaculture.

Introduction

Actinomycetes are distributed extensively in soil and provide many important bioactive compounds of high commercial value, including many of medical importance (Takizawa *et al.* 1993). As an important group of actinomycetes, *Streptomyces* strains could form heat- and desiccation-resistant spores and most of them are nonpathogenic to plants and animals, so *Streptomyces* strains isolated from soil have been regarded as potential biocontrol agents for controlling plant diseases (Emmert & Handelsman 1999).

Actinomycetes can also be isolated from marine sediments, marine water, marine plants and animals. Studies on the microbial diversity by 16S rRNA gene analysis showed that a group of high-GC Gram-positive bacteria (actinomycetes) are dominant in marine sediments (Urakawa *et al.* 1999). Actinomycetes also showed many interesting activities in water, such as degradation of starch and casein and production of antimicrobial agents (Barcina *et al.* 1987; Zheng *et al.* 2000). With these bioactivities, actinomycetes would play an important role in the webs of the marine environment.

The intensive cultivation conditions for marine shellfish larvae may easily cause microbial problems. Marine larvae are small and sensitive, so there is a period with no or low water exchange in the early stage of larval rearing, and this leads to a condition with the high larval densities, the accumulation of debris from dead larvae and high loads of organic matter (Skjermo & Vadstein 1999). Under stress, the marine larvae will lower their feeding activities, the amount of unconsumed feed in the culture pond will increase, and this causes both nutrient enrichment and deterioration of water quality (Sung *et al.* 1999). *Vibrio* spp. are commonly present in seawater and sediments, and the occurrence of *Vibrio* spp. is more frequent in sediment than in water (Bhashkar *et al.* 1998). But many marine *Vibrio* spp. are opportunistically pathogenic bacteria. The proliferation of opportunistically pathogenic *Vibrio* is the major causation of the mortality in waters rich in organic nutrients. In fact, vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in all producing countries. The major species causing vibriosis in shrimp are *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus* (Goarant *et al.* 1999).

Several strategies to control vibriosis have been proposed. For instance, vaccines are being developed to control vibriosis, but they generally cannot be used as a universal disease control measure in aquaculture because they are too time- and labour-intensive. So the addition of substantial amounts of antibiotics and chemotherapeutics remains the method of choice for disease control in many parts of the aquaculture industry. But the abuse of antimicrobial chemicals has led to the occurrence of resistant strains and accumulation of chemicals in aquaculture products. A few studies on antibiotic resistance of *Vibrio* spp. in aquaculture suggested that *Vibrio* spp. are resistant to several antibiotics, such as erythromycin, kanamycin, pencillin G and streptomycin.

In 1990, only 4 antibiotics could be resisted by *Vibrio* spp., but 9 years later, the number of resisted antibiotics had increased to 20 (Eleonor & Leobert 2001). The presence of antimicrobial agents at low concentration through leaching or continued usage may lead to the development of drug-resistant strains and multiple antibiotic resistance (MAR) in bacteria, which may result to resistance transfer to pathogenic bacteria. So developing alternative strategies to the use of antimicrobials in disease control is urgent. One of the successful methods to control vibriosis is the use of probiotics, which are applied in the feed or added to the culture tank or pond as preventive agents against infection by pathogenic. Most probiotics proposed as biological control agents in aquaculture are lactic acid bacteria (*Lactobacillus*, *Carnobacterium* etc.), non-pathogenic *Vibrio* (*Vibrio alginolyticus*, etc.), *Bacillus* strains, and *Pseudomonas* strains (Verschuere *et al.* 2000), but few studies on probiotics consisting of actinomycetes have been reported.

As potential probiotic strains, marine actinomycetes have many advantages: (1) the degradation of macromolecules, such as starch and protein in the culture pond water; (2) the production of antimicrobial agents; (3) the formation of heat- and desiccation-resistant spores.

In order to develop the marine actinomycetes into probiotics to control vibriosis in aquaculture, the actinomycetes were isolated from the marine sediment of shrimp farms, and their activities against *Vibrio* spp. Have been surveyed in this study.

Materials and methods

Media and strains

The AIM medium used for actinomycete isolation contained soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 20 μM, agar 15 g, seawater to 1 l, and 15 μg nalidixic acid were added to inhibit the growth of other bacteria. Siderophore production was tested on solid CAS-agar medium (Clark & Bavoil 1994) and on CAS-substrates with modified Gaus No. 1 medium (MGs). MGs medium contained glucose 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, distilled water to 1 l. The selective medium thiosulphate–citrate–bile salts–sucrose (TCBS) medium was used for cultivation of *Vibrio* spp. (HuanKai Co., Guangzhou, China) and GsB medium was used to screen for actinomycete strains with antimicrobial activity, contained soluble starch 5 g, soybean meal 5 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 20 μM, seawater to 1 l.

The test bacteria included *Vibrio parahaemolyticus* (VP), *V. vulnificus* (VV), *V. anguillarum* (VAN), *V. alginolyticus* (VAL), *V. harveyi* (VH), *V. neresis* (VN), *V. fluvialis* (VF), and all of them were isolated from diseased fish and shrimps in other studies.

Sample collection

Marine sediments were collected from 5 shrimp farms located in Hainan Island, South China, in April 2004. Sediment samples were preserved in ice-box. Samples were processed within 12 h.

Isolation of actinomycetes

To isolate the actinomycetes from the marine sediments, parallel portions of the samples were streaked onto Petri dishes containing AIM medium. Plates were incubated at 28 °C. After incubation for 3 days, colonies of actinomycetes were transferred onto MGs slants.

Identification of actinomycetes

Strains were preliminarily identified according to traditional morphological criteria, including characteristics of colonies on the plate, morphology of substrate and aerial hyphae, morphology of spores, pigment produced and so on (Goodfellow & Cross 1984).

Activities against Vibrio *spp.*

Against a battery of seven pathogenic *Vibrio* strains, the antimicrobial activities of the isolated marine actinomycetes were determined using the double-layer agar method. The actinomycetes were inoculated on Petri dishes containing 15 ml GsB agar medium and incubated at 28 °C for 3 days. Then TCBS agar medium was poured onto the basal layer containing actinomycete colonies, and the *Vibrio* spp. were plated onto the top layer, respectively. The inhibition zones were measured after incubation at 28 °C for 24 h.

Detection of siderophore production activity

Siderophore production was tested on Petri dishes contained CAS-agar (Clark & Bavoil 1994). Actinomycetes were inoculated on CAS-agar plates and incubated at 28 °C for 7 days, and colonies with orange zones were considered as siderophore-producing strains.

Results

Major marine actinomycete flora

Ninety-four marine actinomycete strains were isolated. About 87.5% of the isolates were *Streptomyces* spp., and 12.5% *Micromonospora* spp. The *Streptomyces* isolates were classified into 13 groups, and the cinerogriseus group was the dominant group among the streptomycete isolates (Table 1).

Table 1. The major groups of actinomycete strains isolated and the proportions of antagonistic strains and the siderophore-producing strains, respectively.

Taxon	Number	%	*Vibrio* spp. inhibition		Siderophore production	
			Number	%	Number	%
Groups of *Streptomyces*:						
Cinerogriseus	27	28.7	14	60.9	12	31.6
Griseorubroviolaceus	15	16.0	4	17.4	9	23.7
Albosporus	12	12.8			3	7.9
Globisporus	9	9.6			2	5.3
Flavus	4	4.3	1	4.3	1	2.6
Glaucus	4	4.3			1	2.6
Aureus	4	4.3			1	2.6
Cyaneus	2	2.1			2	5.3
Lavendulae	1	1.1	1	4.3	1	2.6
Roseosporus	1	1.1	1	4.3	1	2.6
Viridis	1	1.1			1	2.6
Griseofuscus	1	1.1	1	4.3	1	2.6
Hygroscopicus	1	1.1			1	2.6
Micromonospora	12	12.8	1	4.3	2	5.3
Total	94	100	23	100	38	100

Activities against pathogenic Vibrio *spp.*

All 94 marine actinomycetes were screened for their antagonism to seven species of *Vibrio*, respectively (Figure 1). A total of 48 actinomycete strains showed activities against pathogenic *Vibrio* strains, and 23 strains (24.5%) could inhibit all the 7 *Vibrio* species (Table 1). The majority of the antagonistic strains belonged to the cinerogriseus group of *Streptomyces*.

Siderophore production on CAS-agar medium

Fifty-four strains (57.4%) could grow on CAS-agar and 38 strains (40.4%) were considered as siderophore producers because orange haloes were formed around the colonies. Most of them belonged to *Streptomyces* and only two strains belonged to *Micromonospora*. All the 13 groups of *Streptomyces* could produce siderophores, but most of them belonged to cinerogiriseus group.

Among all the strains tested, seven strains could not only inhibit the growth of all *Vibrio* spp., but also produce siderophores. They all belonged to *Streptomyces* strains, and could be classified into 5 groups (Table 2).

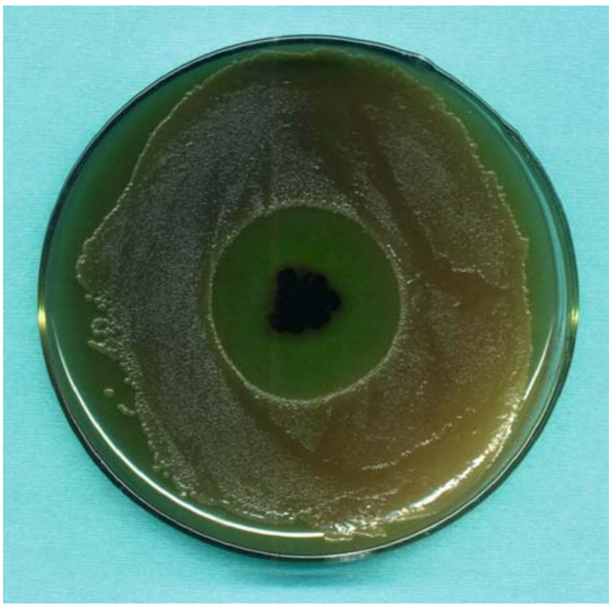

Figure 1. The inhibition zone of marine actinomycetes against *Vibrio* spp.

Discussion

In this study, the majorities of isolated actinomycetes belonged to *Streptomyces* strains and *Micromonospora* strains. It confirms the view about the distribution of actinomycetes that Streptomycetes predominate at shallow depths, and actinoplantes (belonged to the genus *Micromonospora*) increase with increasing depth (Jensen *et al*. 1991). In recent decades, many studies on the antimicrobial activity of the actinomycetes isolated from marine environment have been done, but the results are different. Zheng *et al*. (2000) had found that 43.6% of actinomycetes isolated from the marine environment showed antimicrobial activities, but only 12.8% of the actinomycetes produced antimicrobial metabolites against *Vibrio* spp. In our study, about 51.1% of actinomycete isolates showed antagonistic activities to *Vibrio* spp., particularly, 24.5% of the isolates could inhibit the growth of all the seven *Vibrio* species. The test bacteria in this study were focused on the pathogenic *Vibrio* spp., few of which were tested in the previous studies. It was reported that the components of the medium could influence the antimicrobial activities of actinomycetes (Barcina *et al*. 1987). In this study,

Table 2. Characteristics of siderophore-producing *Streptomyces* strains with antagonistic activity to all seven *Vibrio* species.

Strain No.	Groups	Tested strains[a]							CAS positive
		VH	VN	VF	VAL	VP	VV	VAN	
A03	Cinerogriseus	+ + + +	+ + + +	+ + + +	+ + + +	+ +	+	+ + +	+
A05	Cinerogriseus	+ + + +	+ + + +	+ + + +	+ + + +	+ +	+ +	+ +	+
A26	Griseorubroviolaceus	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + +	+	+ +
A42	Griseorubroviolaceus	+ +	+ + +	+ +	+ + + +	+ +	+ +	+ +	+ + +
A41	Lavendulae	+ + +	+ + +	+ +	+ + +	+ +	+ +	+	+ + +
A45	Roseosporus	+ + +	+ + + +	+ +	+ + +	+ +	+ +	+ +	+ + +
B15	Griseofuscus	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+	–	+ +

Inhibition zone: +, <10 mm; + +, 10–20 mm; + + +, 21–30 mm; + + + +, >30 mm. CAS positive orange hole: +, <5 cm; + +, 5–10 cm; + + +, >10 cm.

[a] For the names of the strains see Materials and methods.

medium consisting of soybean meal was used to screen strains antagonistic to pathogenic *Vibrio* species, for soybean meal is the major component of feed for aquaculture in China. So the actinomycetes screened can grow in the aquaculture environment, even with abundant unconsumed feed in the culture pond, and produce metabolites active against the pathogenic *Vibrio* spp.

Iron is a limiting bioactive metal in seawater and essential for the growth of marine bacteria. The iron concentration in the ocean is low (10^{-17} M) enough to limit the growth of marine microorganisms (10^{-8}–10^{-6} M) (Guerinot 1994). So, marine bacteria have to develop some strategies to acquire iron. The major strategy is the production and utilization of siderophores. The marine actinomycetes that can produce siderophores could adapt to the iron stress conditions in marine environment, even compete for iron with the pathogens. Competition for iron is also a possible mechanism for aquaculture probiotics to control the pathogens. It was reported that during coculture, *Pseudomonas fluorescens* AH2 inhibited the growth of *Vibrio anguillarum* independently of the iron concentration, when the initial level of the siderophore producer was 100 to 1000 times greater than the level of the fish pathogen (Gram *et al.* 1999). So the siderophore-producing *Streptomyces* strains could influence the growth of pathogenic *Vibrio* spp. by competition for iron in marine sediments. Otherwise, *Streptomyces* can form heat- and desiccation-resistant spores, and the probiotic products would be stable in preservation. So the major group of streptomycetes, such as the cinerogriseus group and griseorubroviolaceus group, with antagonistic activity to *Vibrio* spp. and siderophore-producing activities will be promising candidates as aquaculture probiotics.

The relationships between the antagonistic actinomycetes and the pathogenic *Vibrio* spp. in the culture pond environment will be further studied.

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References

Barcina, I., Iriberri, J. & Egea, L. 1987 Enumeration, isolation and some physiological properties of actinomycetes from sea water and sediment. *Systematic and Applied Microbiology* **10**, 85–91.

Bhashkar, N., Setty, T.M.R., Mondal S., Joseph, M.A., Raju C.V., Raghunath B.S. & Anantha C.S. 1998 Prevalence of bacteria of public health significance in the cultured shrimp (*Penaeus monodon*). *Food Microbiology* **15**, 511–519.

Clark, V.L. & Bavoil P.M. 1994 *Methods in Enzymology* 235(A). pp. 315–372. London: Academic Press.

Eleonor A.T. & Leobert, D. de la Pena 2001 Antibiotic resistance of bacteria from shrimp ponds. *Aquaculture* **195**, 193–204.

Emmert, A.B.E. & Handelsman, J. 1999 Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology Letters* **171**, 1–9.

Goarant, C., Merien, F., Berthe, F., Mermoud, I. & Perolat, P. 1999 Arbitrarily primed PCR to type *Vibrio* spp. pathogenic for Shrimp. *Applied and Environmental Microbiology* **65**, 1145–1151.

Goodfellow, M. & Cross, T. 1984 Classification. In *The Biology of the Actinomycetes,* eds. Goodfellow, M., Mordarski, M. & Williams, S.T. pp. 7–164 London: Academic Press. ISBN 012289670X.

Gram, L,. Melchiorsen, J., Spanggaard, B., Huber, I. & Nielsen, T.F. 1999 Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Applied and Environmental Microbiology* **65**, 969–973.

Guerinot, M.L. 1994 Microbial iron transport. *Annual Review of Microbiology* **48**, 743–772.

Jensen, P.R., Dwight, R. & Fenical, W. 1991 Distribution of actinomycetes in near-shore tropical marine sediments. *Applied and Environmental Microbiology* **29**, 1102–1108.

Skjermo, J. & Vadstein, O. 1999 Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* **177**, 333–343.

Sung, H.H., Hsu, S.F., Chen, C.K., Ting, Y.Y. & Chao, W.L. 2001 Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture* **192**, 101–110.

Takizawa, M., Colwell, R.R. & Hell, R.T. 1993. Isolation and diversity of actinomycetes in the Chesapeake bay. *Applied and Environmental Microbiology* **59**, 997–1002.

Urakawa, H., Kita-Tsukamoto, K. & Ohwada, K. 1999 Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology* **145**, 3305–3315.

Verschuere, L., Rombaut, G., Sorgeloos, P. & Verstraete, W. 2000 Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64**, 655–671.

Zheng, Z.H., Zeng, W., Huang, Y.J., Yang, Z.Y., Li, J. & Cai, H.R. Su, W.J. 2000 Detection of antitumor and antimicrobial activities in marine organism associated actinomycetes isolated from the Taiwan Straint, China. *FEMS Microbiology Letters* **188**, 87–91.