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# Characterisation of *Vibrio* and Related Bacteria Associated with Shrimp *Penaeus monodon* Larvae in Indonesia

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## Abstract

To study *Vibrio*-like bacteria associated with *P. monodon* larvae, samples were taken from backyard hatcheries in Jepara, Indonesia, during the dry and rainy seasons (July and December, respectively) of 1995. Bacterial identification of the isolates was performed using BIOLOG and FAME fingerprints. Dominant bacterial strains were identified as *V. alginolyticus, V. damsela,* and *V. harveyi,* while some of the non-*Vibrio* species were identified as *Brevundimonas*-like bacteria. *Vibrio* species were found at different larval stages, during both the dry and rainy seasons and in both diseased and healthy larvae. The study supported the idea that *Vibrio* species are part of the residential microflora in *P. monodon* larvae and that the growth of *V. alginolyticus* and *V. harveyi* is promoted by water quality conditions during the rainy season.

## Introduction

The Indonesian shrimp farming industry has grown rapidly over the last 20 years. In fact, Indonesia even became the second highest shrimp producing country in the world in 1994 (Chamberlain 1991; Rosenberry 1997). However, failures in shrimp production occurred. Shrimp production of 140,000 metric tonnes in 1994 dropped to 90,000 metric tonnes in 1996 (Rosenberry 1997). Diseases, caused mostly by bacteria, viruses, and fungi,

were believed to be the cause of production problems which occured in hatcheries and growout ponds (Ruangpan and Kitao 1991).

Gram negative bacteria which are predominant in marine environments (Farmer and Hickman-Brenner 1992) are also present as normal flora in cultivated and wild penaeid marine shrimp (Lightner et al. 1992). Negative interaction between shrimp larvae and bacteria often causes diseases, which lead to rapid mass mortality of the larvae. While a number of bacterial species have been implicated as agents causing infections and diseases in penaeid shrimp, reports of infections by *Vibrio* species are by far the most numerous (Lightner 1988; Lightner 1993).

In order to characterise *Vibrio*-like bacteria in Indonesian marine shrimp larviculture, bacterial strains were isolated from *Penaeus monodon* larvae derived from six private (back yard) shrimp hatcheries on the Java island (around Jepara). This is the most developed shrimp larviculture area in Indonesia (Yap 1990; Sunarjanto et al. 1992). In order to verify whether there is a seasonal variation in the bacterial microbiota of these hatcheries, samples were taken in July and December 1995, during the dry and rainy seasons, respectively.

## Methods

### *Collection of samples and isolation of dominant bacteria*

Either one, two, or three samples were taken from each hatchery. Samples of 10 shrimp larvae of the same age were taken in a sterile beaker from one larval culture tank, using a sterile pipette transfered to a sterile microwell containing 10 ml sterile saline solution (SS 1.5% w/v NaCl) for rinsing. Rinsing was performed three times afterwhich, the larvae were immediately macerated and homogenised in a sterile glass potter containing 1 ml saline solution. Serial dilutions were made up to $10^{-3}$ using saline solution.

The larval suspensions were plated on Thiosulphate Citrate Bile Sucrose Agar (TCBS, Difco), a selective medium for *Vibrio* and related bacteria (Nash et al. 1992), and incubated at 27 ºC for 24-48 hours.

### *Purification and storage of the isolates*

According to the colony shape and morphology, the three most dominant colony types of each sample were purified on TCBS and incubated at a temperature of 27 ºC. The pure strains were kept on TCBS and transferred at weekly intervals to marine agar (MA) slants. The MA was made from 38 gr Marine Broth (MB, Difco) and 25 gr technical agar diluted with aquadest to be 1 liter.

At the Laboratory of Microbiology, University of Ghent, Belgium, the strains were plated on MA and strain purity was checked. Pure strains were suspended in Marine Broth (MB, Difco) supplemented with 20% (w/v)

glycerol and stored in a liquid nitrogen container at -140 ºC (Verdonck et al. 1997).

### *Characterisation of the isolates*

A gram staining test was performed to identify gram negative and gram positive strains, using the method described by Smibert and Krieg (1981), and only the gram negative strains were used in this work. After the initial test, the gram negative strains were characterized using BIOLOG metabolic fingerprints (Austin et al. 1995a; Verdonck et al. 1997; Vandenberghe et al. 1997).

Identification was performed by comparing the BIOLOG profiles with a database of BIOLOG fingerprints of approximately 850 *Vibrio* strains and 37 *Vibrio* type strains, mostly of aquaculture or marine origins. The comparison used numerical analysis which was calculated using the Pearson product moment correlation coefficient. The strains were then grouped according to the unweighted pair-group cluster analysis (Vauterin et al. 1995).

Unidentified strains were further analysed using cellular Fatty Acid Methyl Esters (FAME), as described by Verdonck et al. (1997). The FAME profiles were compared to a laboratory database of reference strains for identification.

## Results

### *Identification of strains*

Fifty six gram negative bacterial strains were collected from the larvae of penaeid shrimp (*Penaeus monodon*) in Jepara, Indonesia; 25 strains were isolated during the dry season and 31 strains during the rainy season.

Based on BIOLOG identification, two groups of bacteria were distinguished; *Vibrio* species (39 strains) and unidentified gram negative bacteria (17 strains). When the FAME procedure was used for identification of the unidentified bacteria, 10 strains (unidentified cluster STD3-236 strains) belonged to *Brevundimonas*-like bacteria, while 7 strains remained unidentified. *Vibrio* species were found in the dry and rainy seasons, while *Brevundimonas*-like bacteria were found only in the dry season. Dendrogram of bacterial strains derived by cluster analysis of BIOLOG data are presented in Figures 1 and 2.

The *Vibrio* group comprised three species; *V. alginolyticus*, *V. damsela*, and *V. harveyi*, and one group of unidentified *Vibrio* strains. *V. harveyi* was the most frequently isolated followed by *V. alginolyticus* and *V. damsela*. The first two species tended to be associated with all the larval stages, while *V. damsela* was isolated only from the post larval stages. All of the identified *Vibrio* bacteria were associated with both healthy and diseased larvae.

Distribution of the bacterial strains during the dry and rainy seasons is presented in Table 1.

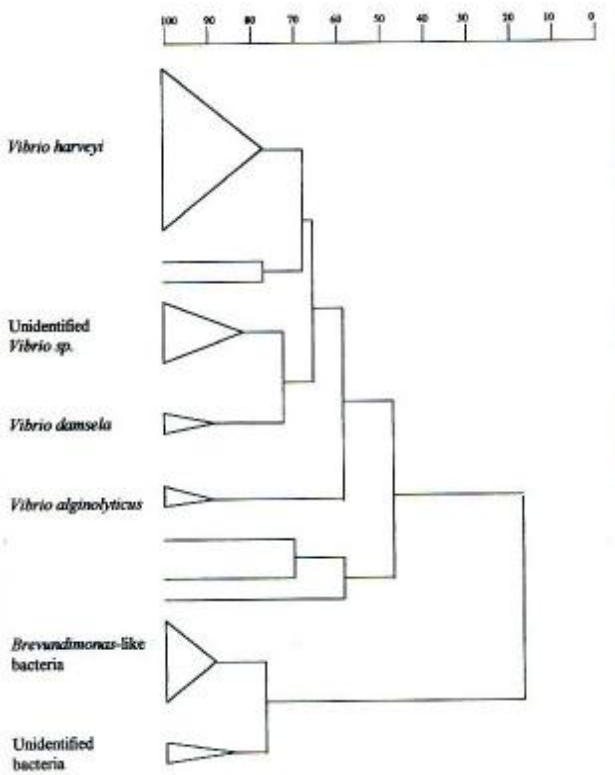


Fig. 1. Dendogram of isolated strains together with some reference strains obtained after numerical analysis of BIOLOG profiles for the isolation carried out during the dry season.

Table 1. List of members and types of bacteria isolated during the dry (D) and rainy (R) seasons.

| Bacteria | Sampling seasons | Number of isolates |
|---|---|---|
| Vibrios bacteria: | | |
| *Vibrio alginolyticus* | D | 3 |
| | R | 9 |
| *Vibrio harveyi* | D | 4 |
| | R | 11 |
| *Vibrio damsela* | D | 4 |
| | R | - |
| *Vibrio* spp. | D | 3 |
| | R | 5 |
| Non vibrios bacteria: | | |
| *Brevundimonas*-like bacteria | D | 10 |
| | R | - |
| Unidentified bacteria | D | 1 |
| | R | 6 |

Notes :Most of the strains were isolated from the post larval stage. Only one *Vibrio* strain was isolated from the zoeal stage and 3 *Brevundimonas*-like strains from the mysis stage.

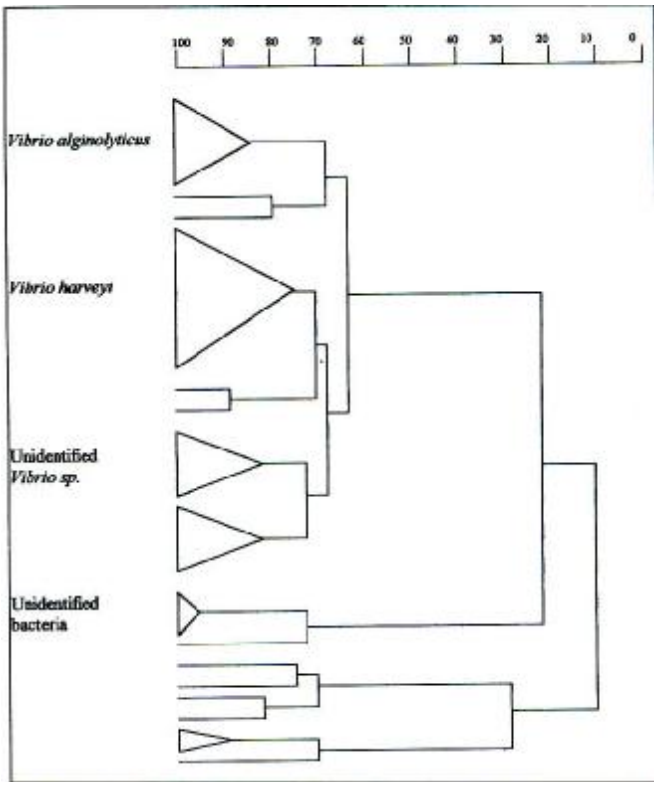


Fig. 2. Dendogram of isolated strains together with some reference strains obtained after numerical analysis of BIOLOG profiles for the isolation carried out during the rainy season.

### *Seasonal variations*

In order to examine the seasonal effects on the occurrence of the isolated bacteria in growing shrimp larvae from hatcheries, percentage occurrence of all the isolates was calculated in Table 2. Vibrio strains constituted the majority of the isolated bacteria (69.6%) which were isolated during both the dry and rainy seasons. Nevertheless, the numbers during the rainy season were higher than during the dry season. Two vibrio species, *V. alginolyticus* and *V. harveyi*, were dominant. They constituted 64.52% of the bacteria isolated during the rainy season, but only 28.0% during the dry season. Interestingly *V. harveyi* strains were isolated from both healthy and diseased larvae during the dry season, but they were isolated from the diseased larvae only during the rainy season.

Other species, *V. damsela* and *Brevundimonas*-like bacteria, were isolated only during the dry season. *Brevundimonas*-like bacteria constituted the majority (58.8%) of the non-*Vibrio* bacteria.

## Discussion

This work showed that *Vibrio* species were commonly associated with shrimp larvae. They accounted for 56.0% and 80.6% of all the strains isolated on TCBS during the dry and rainy seasons, respectively.

Table 2. Occurrence of bacteria in the dry and rainy season samples.

| Name of bacteria | Sampling | | | | Total | |
|---|---|---|---|---|---|---|
| | Dry season | | Rainy season | | | |
| | N | % | N | % | N | % |
| *Brevundimonas*-like | 10 | 40.0 | - | - | 10 | 17.9 |
| *Vibrio harveyi* | 4 | 16.0 | 11 | 35.5 | 15 | 26.8 |
| *Vibrio alginolyticus* | 3 | 12.0 | 9 | 29.0 | 12 | 21.4 |
| *Vibrio damsela* | 4 | 16.0 | - | - | 4 | 7.1 |
| *Vibrio* spp. | | | | | | |
| - cluster STD3-194 | 1 | 4.0 | - | - | 1 | 1.8 |
| - cluster STD3-348 | 1 | 4.0 | 2 | 6.45 | 3 | 5.3 |
| - cluster STD3-565 | - | - | 2 | 6.45 | 2 | 3.6 |
| - cluster STD3-571 | 1 | 4.0 | 1 | 3.2 | 2 | 3.6 |
| Unidentified | | | | | | |
| - cluster STD3-199 | 1 | 4.0 | - | - | 1 | 1.8 |
| - cluster STD3-556 | - | - | 4 | 13.0 | 4 | 7.1 |
| - cluster STD3-559t1 | - | - | 1 | 3.2 | 1 | 1.8 |
| - cluster STD3-580 | - | - | 1 | 3.2 | 1 | 1.8 |
| Total | 25 | 100.0 | 31 | 100.0 | 56 | 100.0 |

Notes: N is the number of isolates.

Although there are more than 30 currently recognized *Vibrio* species (Farmer 1992), only three species were clearly identified in this work. *Vibrio* species are considered as part of the normal flora of sea water and can invade marine animals (Olsen et al. 1995). Infection with one or more species of *Vibrio* is called vibriosis, which may sometimes be manifested as systemic diseases in both humans and animals (Egidius, 1987).

Our results show that *Vibrio* strains came from different larval stages and from the larvae of different health status. These findings support the idea that *Vibrio* species are part of the normal bacterial flora for marine penaeid larvae and that they may cause diseases as opportunistic pathogens under stress conditions. Stress, which either lowers the resistance of the host or enhances the effects of pathogens on the host, is an important factor in the disease process (Lightner et al. 1992).

Vibriosis is feared by hatchery managers and tended to breakout in Japara (Indonesia) during the rainy season, between November and February (Prayitno and Latchford, 1995). Heavy rains in Jepara and in the surrounding area reduce salinity, increase pH, and lower the temperature of hatchery intake water. Changing water conditions have been supposed to cause excessive growth of *Vibrio* bacteria (Sunaryo and Mariam, 1986). Since the occurrence of *Vibrio* was higher during the rainy season than during the dry season, it appeared that the rainy season promoted the occurrence and excessive growth of these bacteria particularly *V. harveyi* and *V. alginolyticus*.

*V. harveyi* has been described as a marine pathogenic luminous or nonluminous bacterium (Farmer and Hickman-Brenner 1992). Infections of luminescent bacteria can cause diseases leading to weakness and death of

the animal host. Mortality of *Penaeus monodon* larvae infected with luminescent bacteria has been reported in some hatcheries in Indonesia, in Thailand, and in the Philippines (Lightner et al. 1992). Experimental infections of *V. harveyi* performed by Lavilla-Pitogo et al. (1990) and Prayitno and Latchford (1995) resulted in high mortalities of *P. monodon* larvae. *V. harveyi*, according to Song et al. (1990), is rather endemic and may become an opportunistic pathogen under special conditions. In this work, *V. harveyi* strains were found only among diseased larvae during the rainy season.

*V. alginolyticus*, the second most frequently isolated bacterium here (21.4%), is very common in the marine environment and is a normal part of the microflora of pond and raceway-reared shrimp (Lightner 1988). Its effect on the growing larvae is rather unclear. It has been reported to be a pathogen for *P. monodon* larvae (Nash et al. 1992). Nevertheless, experimental work conducted by Austin et al. (1995b) showed that the bacterium could be used as a probiotic for limiting the development of fish pathogens such as *Aeromonas salmonicida, V. anguillarum* and *V. ordalli.*

*V. damsela* is a pathogenic bacterium that can cause lesions in both warm and cold blooded marine animals (Fouz et al. 1995). This bacterium, together with *V. harveyi* and *V. alginolyticus*, was implicated in disease outbreaks in shrimp larvae during the dry season in Jepara, since it was not found during the rainy season. Nevertheless, it appeared as a minority (7.4%) of the total isolated strains.

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## References

Austin, B., M. Alsina, D.A. Austin, A.R. Blanch, P.A.D. Grimont, J. Jofre, S. Koblavi, J.L. Larsen, K. Pedersen, T. Tiainen, L. Verdonck and J. Swings. 1995a. Identification and Typing of *Vibrio anguillarum* : A comparison of methods. System. Appl. Microbiol 18: 285-302.

Austin, B., L.F. Stuckey, P.A.W. Roberston, I. Effendi and W. Griffith. 1995b. A probiotic strain of *Vibrio alginolyticus* effective in reducing deseases caused by *Aeromonas salmonicida, Vibrio anguillarum,* and *Vibrio ordalii.* J. Fish Diseases 18: 93-96.

Chamberlin, G.W. 1991. Shrimp farming in Indonesia-2; Seedstock Production. World Aquaculture 22(3): 51-57.

Egidius, E. 1987. Vibriosis : Pathogenicity and pathology. A review. Aquaculture 67: 15-28.

Farmer III, J.J. 1992. The family of Vibrionaceae. In : The Prokaryotes (eds. A. Balows, H.G. Truper, M. Dworkin, W. Harder and K.H. Schleifer), 2nd edition, Vol. III, pp. 2938-2951. Springer-Verlag. New York.

Farmer III, J.J. and F.W. Hickmann-Brenner. 1992. The genera *Vibrio* and *Photobacterium*. In : The Prokaryotes (eds. A. Balows, H.G. Trupper, M. Dworkin, W. Harder and K.H. Schleifer). 2nd edition, Vol. III, pp. 2952-3011. Springer-Verlag. New York.

Fouz, B., B. Novoa, A.E. Toranzo and A. Figueras. 1995. Histopatological lesions caused by *Vibrio damsela* in cultured turbot, *Scophthalmus maximus* (L.) : inoculations with live cells and extracellular products. Fish Diseases 18 : 357-364.

Lavilla-Pitogo, C.R., M.C.L. Baticados, E.R. Cruz, Lacierda and de la Pena. 1990. Occurrence of luminous bacterial diseases of *Penaeus monodon* larvae in the Philippines. Aquculture 91: 1-13.

Lightner, D.V. 1988. Vibrio disease of Penaeid shrimp. In : Disease Diagnosis and Control in North American Marine Aquaculture (eds. C.J. Sidermann and D.V. Lightner), pp. 42-47. Elsevier Publishers, Amsterdam.

Lightner, D.V., T.A. Bell, R.M. Redman, L.L. Mohney, J.M. Natividad, A. Rukyani and A. Poernomo. 1992. A review of some major diseases of economic significance in Penaeid prawns/shrimps of the Americans and Indopacific. In : Disease in Asian Aquaculture I (eds. M. Shariff, R.P. Subasinghe and J.R Arthur). Proceedings of the first Symposium on Disease in Asian Aquaculture, Bali, Indonesia, pp. 57-80. Fish Health Section, Asian Fisheries Society. The Philippines.

Lightner, D.V. 1993. Diseases of cultured shrimp. In CRC Handbook of Mariculture (ed. P.V. McVey), pp. 393-486. CRC Press, Boca Raton, Florida, USA.

Nash, G., C. Nithimathachoke, C. Tungmandi, A. Arkarjamorn, P. Prathanpipat and P. Ruamthaveesub. 1992. Vibriosis and its control in pond-reared *Penaeus monodon* in Thailand. In : Disease in Asian Aquaculture I (eds. M. Shariff, R.P. Subasinghe and J.R Arthur). Proceedings of the first Symposium on Disease in Asian Aquaculture, Bali, Indonesia, pp. 143-155. Fish Health Section, Asian Fisheries Society. The Philippines.

Olsen , J.E., S. Aabo, W. Hill, S. Notermans, K. Wernars, P.E. Granum, T. Popovic, H.N. Rasmussen and O. Olsvik. 1995. Probes and polymerase chain reaction for detection of foodborne bacterial pathogens. Int. J. Food Microbiology 28 : 1-78.

Prayitno, S.B. and J.W. Latchford. 1995. Experimental infection of crustacean with luminous bacteria related to *Photobacterium* and *Vibrio*. Effect of salinity and pH on infectiosity. Aquaculture 132 : 105-112.

Rosenberry, B. 1997. World shrimp farming. Annual Report. Shrimp News International, San Diego, California, USA. 164 pp.

Ruangpan, L. and T. Kitao. 1991. *Vibrio* bacteria isolated from black tiger shrimp, *Penaeus monodon* Fabricius. J. Fish Diseases 14 : 383-388.

Smibert, R.M. and N.R. Krieg. 1981. General characterization, p. 409-443. In : Manual of methods for general microbiology.

Song, Y.L., W. Cheng, C.H. Shen, Y.C. Ou and H.H. Sung. 1990. Occurrence of *Vibrio vulnificus* infections in cultured shrimp and eel in Taiwan. Proceedings of ROC-JAPAN Symposium of Fish Diseases, September 1990, p. 172-179. Japan.

Sunaryanto, A., Anindiastuti and I.S. Djunaidah. 1992. Implementation of small-scale shrimp hatchery technology. Bull. Brackishwater Aqua. Dev. Cent. 9(1) : 40-47.

Sunaryanto, A. and A. Mariam. 1986. Occurrence of a phatogenic bacteria causing luminescence in penaeid larvae in Indonesian hatcheries. Bull. Brackishwater Aquaculture Development Centre. 8(2) : 64-70.

Vandenberghe, J., L. Yun, L. Verdonck, H.S. Xu and J. Swings. 1997. Vibrios associated with *P. chinensis* larvae in Chinese shrimp hatcheries (in press). Aquaculture.

Vauterin, L., B. Hoste, K. Kersters and J. Swings. 1995. Reclassification of Xanthomonas. Int. J. Syst. Bacteriol 45 : 472-489.

Verdonck, L., L. Grisez, E. Sweetman, G. Minkoff, P. Sorgeloos, F. Ollevier and J. Swings. 1997. Vibrios associated with routine productions of *Brachionus plicatilis*. Aquaculture 149 : 203-214.

Yap, W.G. 1990. Backyard hatcheries take off in Jepara. Infofish International 2 : 42-47.

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