Characterization and experimental infection of *Flexibacter maritimus* (Wakabayashi et al. 1986) in hatcheries of post-larvae of *Litopenaeus vannamei* Boone, 1931

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(With 2 figures)

Abstract

A preliminary study to characterize filamentous bacteria, whose presence is related to high mortality of *Litopenaeus vannamei* larvae cultured in Santa Catarina State, Brazil, is reported. The extract of infected larvae was diluted in different concentrations, cultured in marine agar (Difco™, Marine Agar 2216) and incubated at 30 °C for 48 hours. The biochemical characterization included hydrolytic reactions of starch, gelatin and tyrosine, growth in TCBS agar, growth in 0 and 37‰ salinity, pigment production in tyrosine agar, production of H_2S, nitrate reduction, congo red reaction, oxidase and catalase. The isolated bacteria belong to the species *Flexibacter maritimus*, Gram-negative bacilli of 0.4-0.5 µm width and 15 µm length. Experiments were carried out on pathogenicity of *F. maritimus* in post-larvae of *L. vannamei*. Survival and symptoms in *L. vannamei* post-larvae 24 hours after inoculation with *F. maritimus* and its growth in marine agar were evaluated. Mortality was detected around 92,5% as well as symptoms like melanized lesions in several parts of body, discolouration of gills, bad formation of appendages and of the last abdominal segment, low motility and feeding reduction. The experimental infection results suggested that isolated bacteria of the genus Flexibacter are pathogenic to the shrimp *Litopenaeus vannamei* post-larvae.

Keywords: Litopenaeus vannamei, hatchery, *Flexibacter maritimus*, experimental infection.

Caracterização e infecção experimental de *Flexibacter maritimus* em pós-larvas de *Litopenaeus vannamei*

Resumo

Neste trabalho, relata-se um estudo preliminar para caracterizar bactérias filamentosas associadas a elevadas mortalidades de larvas e pós-larvas cultivadas no Estado de Santa Catarina, Brasil. Um extrato de larvas infectadas foi diluído em diferentes concentrações, cultivado em Agar Marine (Difco™, Marine Agar 2216) e incubado a 30° C por 48 horas. A caracterização bioquímica inclui reações de amido, gelatina e tirosesine, crescimento em Agar TCBS,crescimento a 0 e 37‰ de salinidade, produção de pigmentos em Agar Tirosine, produção de H_2S, redução de nitrato, reação vermelho congo, oxidase e catalase. A bactéria isolada apresentou características de *Flexibacter maritimus*, um bacilo Gram negativo de 0,4-0,5 µm de largura e 15 µm de comprimento. Os experimentos foram realizados sobre a patogenicidade de *F. Maritimus*, em pós-larvas de *L. vannamei*. Foram avaliados a sobrevivência e os sintomas das pós-larvas 24 horas após a inoculação com *F. Maritimus*, além do seu crescimento em Agar Marine. Foram encontradas mortalidades de 92,5% e sintomas próprios da enfermidade, tais como lesões melanizadas em várias partes do corpo, descoloração das brânquias e diminuição da motilidade e da alimentação. Com base nos resultados da infecção experimental, pode se concluir que a bactéria isolada do gênero Flexibacter é patogênica para pós-larvas de *L. vannamei*.

Palavras-chave: Litopenaeus vannamei, pós-larvas, *Flexibacter maritimus*, infecção experimental.

1. Introduction

It has been noted that more than 60% of the mortalities in mysis 3 and post-larva 1 in the hatcheries of *Litopenaeus vannamei* in Santa Catarina state are related to the presence of a great number of filamentous bacteria. Dead larvae presented melanized lesions in several parts of body, discolouration of gills, bad formation of appendages and of the last abdominal segment. Surviving larvae, besides the lesions described above, also presented reduction in the growth, metamorphosis, motility and feeding. The occurrence of the bacteria was confirmed through simple microscope observation at 100x.

Some bacteria take part of the natural flora of marine organisms and their ecosystems (Yasuda and Kitao, 1983). Different opportunistic bacteria have been reported to cause severe losses in the shrimp industry due to mortality, tissue lesions (necrosis), bad formation, reduced growth and larval metamorphosis (Bower et al., 1994).

According to Lightner (1996), shrimp diseases caused by filamentous bacteria are known worldwide. All shrimp species can be affected by these microorganisms. The most commonly found genera are *Cytophaga* sp., *Flavobacterium* sp., *Flexibacter* sp., *Leucothrix* sp. and *Thiotrix* sp.. It has also been reported that high bacterial infection rates cause necrosis and serious histological changes in gill tissues in shrimp larvae, affecting the respiratory system, and physiology as a whole (Kampfer, 1997; Amrane and Prigent, 1998).

Bacteria often occur in large numbers, forming bacterial nets that attach to necrotized surfaces, suggesting that powerful exotoxins are produced to attenuate host defense responses (Baxa et al., 1988). In the case of filamentous bacteria, they create a perfect biofilm to which other microorganisms or organic matter are attached, and when this happens in shrimp gills, it affects their respiration (Kitatsuji et al., 1996).

Diagnosis can be done through microscopic examination at 100x of shrimp body surface and gills. This methodology reveals presence of bacteria's filaments. Histological techniques can also be used for diagnosis together with microbiological techniques (Lightner, 1996; Skjermo and Vadstein, 1999).

Therefore, the aim of this study was to characterize and identify, based on morphological description, culture characteristics and biochemical response of filamentous bacteria associated with mortality and several lesions caused to larvae of *L. vannamei* and to demonstrate the possible pathogenicity of *F. maritimus*.

2. Material and Methods

2.1. Animals

Shrimp post-larvae from the Marine Shrimp Laboratory of the Federal University of Santa Catarina (UFSC) were used, with melanized lesions in several body parts, discolouration of gills, bad formation of appendages, reduced growth, delaying in metamorphosis of larval stages, reduced motility and feeding.

2.2. Isolation and characterization

Larvae samples were weighed in digital scale (0.001 g) and 1 mL of sterile seawater was added in a 1:1 ratio, following the relation with body weight. Larvae were then macerated until complete trituration. Using a 1 mL micropipette, macerate samples were diluted in solutions containing concentrations of 10^0, 10^{-1}, 10^{-2} CFU.mL^{-1} in order to be scattered in marine agar (Difco™, Marine Agar 2216). Plates were incubated for 48 hours in bacteriological stove at 30 °C.

Isolation of characteristic colonies was done using the streaked plate method. Pure cultures were confirmed through microscope observation using the gram stain method. The pure cultures were progressively diluted and inoculated in tubes with solidified marine agar in order to store isolated strain and for further biochemical characterization.

The biochemical characterization was as follows: starch, gelatin and tyrosine hydrolysis reactions, growth in TCBS agar, growth in 0 and 37‰ salinity, pigment production in tyrosine agar, production of H_2S, nitrate reduction, congo red reaction, oxidase, and catalase. These reactions were done according to Lewin and Lounsbery (1969), Wakabayashi et al. (1986), and Chen and Henry-Ford (1995).

2.3. Experimental infection

The present study was carried out at the Laboratory of Diagnosis and Pathology in Aquaculture, Aquaculture Departament, UFSC. Healthy post-larvae of *L. vannamei* with average of 0.8 ± 0.12 cm length from Marine Shrimp Laboratory, UFSC, and a strain of *F. maritimus* were used for the experiment. This strain was isolated during an outbreak of post-larvae mortality.

Purity-confirmed strain of *F. maritimus* was used for the preparation of the inoculum. Purity of isolated culture was confirmed by identification of white clear rhizoid shaped colony and also through microscopic observation using gram staining method. The strain was cultured in BHI (BHI Broth - Oxoid, England) and incubated for 24 hours at 30 °C with continuous mixing. *Flexibacter maritimus* inoculum concentration after incubation was 1.2×10^8 CFU mL^{-1}.

The experiment was divided into two groups of 4 replicates in a completely random design. Each experimental unit consisted of 1 L flask with 50 shrimp post-larvae. Water in the flasks of the first treatment was inoculated with 6 mL.L^{-1} of *F. maritimus* with the inoculum. The water in the flasks of the second treatment was inoculated with 6 mL of sterile culture medium (BHI).

The experiment was evaluated 24 hours after inoculation with *F. maritimus*. Survival was observed, as well as clinical signs of the disease in the post-larvae under optic microscope. Post-larvae were cultured in plates in order to detect the presence of *F. maritimus*. Post-larvae from treatments were macerated in 5 mL of sterile salt water. Re-isolation of colonies from that solution was done using streaked plate method, followed by incubation at 30 °C

for 48 hours in marine agar (Difco™, Marine Agar 2216). Survival values were submitted to the *t*-test check for significant differences between treatments ($P < 0.05$).

3. Results and Discussion

As previously mentioned, post-larvae used for filamentous bacteria isolation presented symptoms directly related to mortality, necrosis, bad formation, reduced growth, metamorphosis, feeding and apathy, which were symptoms also found by Bower et al. (1994), when they described the effects of filamentous bacteria in penaeid shrimp.

When observed under microscope, it was possible to distinguish long and thin bacilli (Figure 1) attached to melanized lesions, and hemorrhagic spots on the exoskeleton.

Colonies that grew after 48 hours incubation at 30 °C in marine agar were rhizoid in shape and creamy in color. They presented Gram-negative filaments with 0.4-0.5 µm wide and 15 µm long. When left to grow for a longer period, they became smaller, with many units forming spores (Figure 2), a characteristic also found by Chen and Henry-Ford (1995).

Results of biochemical characterization agreed with those of Lewin and Lounsbery (1969), Wakabayashi et al. (1986), and Chen and Henry-Ford (1995) (Table 1). Biochemical tests, morphological and phenotypical characteristics, and comparisons done according to Austin and Austin (1987), suggested that the bacteria belongs to the species *F. maritimus*. According to Bernardet and Grimont (1989), these results indicate that preliminary tests to identify *F. maritimus* from outbreak mortality do not require sophisticated materials or equipment.

The genus *Flexibacter* was described by Wakabayashi et al. (1986) as a pathogen in marine fish (Austin and Stobie, 1991; Ostland et al., 1997; Soltani et al. 1995). According to Baxa et al. (1986), these bacteria affect several species, such as *Pagrus major* (Temminck and Schlegel, 1843), *Acanthopagrus schlegeli* (Bleeker, 1854), *Oplegnathus fasciatus* (Temminck and Schlegel, 1844) and *Paralichthys olivaceus* (Temminck and Schlegel, 1846). Toxins produced by these bacteria were verified by Baxa et al. (1988) in in vivo and in vitro tests.

Bacteria isolated from *L. vannamei* larvae were compared to isolated strains coming from other countries and from different origins, in order to determine biochemical and physiological similarities. Isolated bacteria are virtually identical to San Diego strains (SD2), except for growth in TCBS agar and in 35‰ salinity. When compared to *Flexibacter sancti* (Lewin, 1969) strains, they differ in nitrate reduction, starch and tyrosine hydrolysis, pigment production in tyrosine agar and growth in TCBS agar.

Recent studies using new methodologies have shown the existence of different serological groups closely associated with different species (Avendaño-Herrera et al., 2004). Several published articles detail homogeneity of *F. maritimus*, considering a homogeneous taxonomy based on different biochemical and physiological characteristics, which seems to explain the differences found

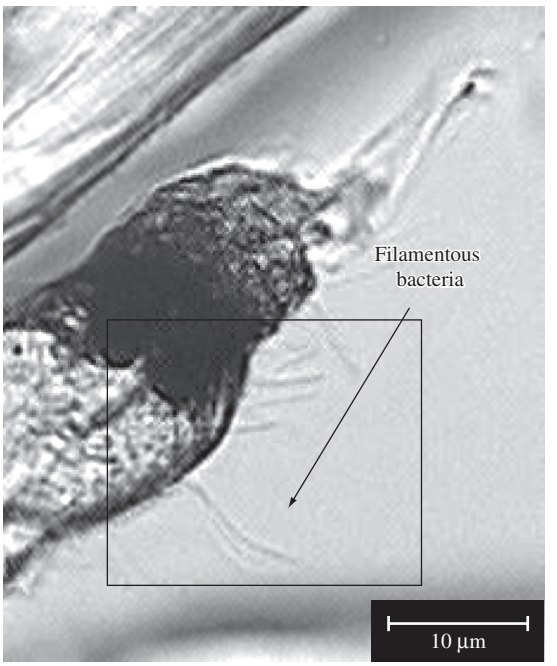

Figure 1. Filamentous bacteria attached to necrosis in pleopode of *Litopenaeus vannamei* larva (mysis 3).

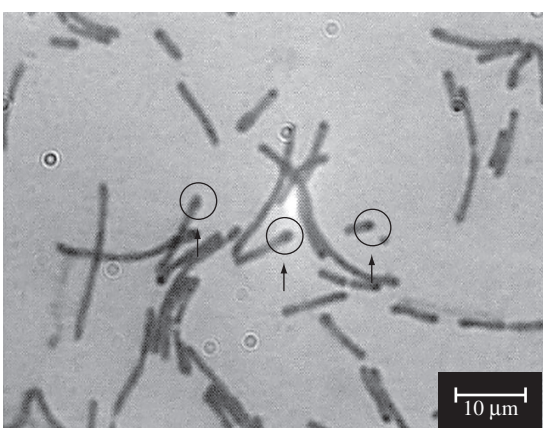

Figure 2. Photomicrograph of filamentous bacteria with spores cells stained by gram method.

in our research (Wakabayashi et al., 1984; Baxa et al., 1986; Bernardet and Grimont, 1989; Bernardet et al., 1990 and Chen et al., 1995).

It is presumed that the presence of highly virulent strains of *Flexibacter* sp. is associated with the presence of organic matter dissolved in water, which leads to the development of the disease. This is also correlated to *Flavobacterium columnare* (Bernardet and Grimont, 1989), according to Hanson and Grizzle (1985), since water quality is directly related to their appearance. Maintenance of good water quality, use of adequate and balanced diets, and prophylactic treatments (e. g. formalin) can result in low filamentous bacteria levels in the system (Skjermo and Vadstein, 1999). In fact, the higher the water exchange rates in culture ponds

Table 1. Biochemical characteristics of filamentous bacteria isolated in *Litopenaeus vannamei* hatchery and reference strains. (+) positive reactions, (–) negative reactions and NT not tested. SD2 – *Flexibacter maritimus* type strain isolated from *Atractoscion nobilis* (Ayres, 1860), San Diego (EUA).

	Isolated bacteria				
Test	***Cytophaga aquatilis*** [a]	***Flexibacter maritimus***[a]	**ATCC 23092**[b]	**SD2**	**Isolated strain**
Congo red	NT	NT	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	+	+	+	+
Nitrate Reduction	+	+	-	+	+
H_2S Production	-	-	-	-	-
Hydrolysis of:					
Starch	+	-	+	-	-
Gelatin	+	+	+	+	+
Tyrosine	+	+	-	+	+
Pigment Production in Tyrosine Agar	NT	NT	-	+	+
Growth:					
0‰ salinity	NT	NT	+	-	-
35‰ salinity	NT	NT	+	-	+
TCBS	NT	NT	+	+	-
Sucrose	+	NT	NT	-	-

[a] Strains characterization done according to Austin and Austin (1987).
[b]*Flexibacter sancti* type strain.

or better water disinfection methods, the lower the concentration of bacteria (Lightner, 1996).

Survival in the control treatment (65%) was higher ($P < 0.05$) than in the treatment inoculated with *F. maritimus* (7.5%). Control treatment still presented high mortality, probably due to the inoculation of culture medium, which may have caused proliferation of other pathogenic bacteria or possible decay of water quality.

Inoculation with *F. maritimus* strain caused clinical signs of pathogenicity directly correlated to mortality. Every post-larvae from the inoculated treatment presented melanized tissue lesions (necrosis) and hemorrhagic spots on the surface, which did not occur in post larvae of the control. Necrosis was also described by Bower et al. (1994), demonstrating the bacteria's dermonecrotic ability and, if not treated or prevented, it can cause 80% mortality in larvicultures.

In inoculated shrimp post-larvae, *F. maritimus* were observed on gills and around lesions, leading to necrosis and physical changes in gills, thus impairing the respiratory system. Also observed was the formation of bacterial nets adherent to necrosis, suggesting the production of exotoxins that promote host response (Lumsden et al., 1996; Bertolini. et al., 1994).

All life stages of penaeid shrimp can be infested by *F. maritimus* and other filamentous bacteria. However, the presence of those bacteria is common and, depending on the degree of infestation, gills can be affected but without causing immediate mortality (Bower et al., 1994). Nevertheless, in post-larval stage this infestation may cause great levels of mortality as shown in this study.

It was possible to re-isolate *F. maritimus* only from dead post-larvae treatment inoculated with the pathogenic bacterium strain. Purity was confirmed under microscopic observation of characteristic colonies. After Gram staining, Gram negative bacteria with stems 0.4-0.5 µm wide and 15 µm long (Chen and Henry-Ford, 1995) were observed.

Colonization of gills by filamentous bacteria has already been described by Powell et al. (2004) in fish (*Salmo salar* (Linnaeus, 1758)), which presented clinical signs of "black gill disease", morbidity and mortality 24 hours after inoculation with *Tenacibaculum maritimum* (Wakabayashi et al. 1986) in the gills.

Effective control of filamentous bacteria loads in the system can be achieved by maintaining good water quality, use of balanced diets and prophylactic treatments (Skjermo and Vadstein, 1999).

The results in this experiment with biochemical characterization can conclude that the isolated bacteria belongs to the species *Flexibacter maritimus*, Gram-negative bacilli of 0.4-0.5 µm width and 15 µm length, and thus it shows that *Flexibacter maritimus* strain is pathogenic and can cause massive mortality of *L. vannamei* post-larvae.

References

AMRANE, A. and PRIGENT, Y., 1998. A new turbidimetric device for on-line monitoring of growth of filamentous microorganisms. *J. Microbiol. Methods*, vol. 33, no. 1, p. 37-43.

AUSTIN, B. and AUSTIN, DA., 1987. *Fish pathogens diseases in farmed and wild. Chichester*, Ellis Horwood, UK, p. 196-224.

AUSTIN B. and STOBIE M., 1991. Recovery of yellow-pigmented bacteria from dead and moribund fish during outbreaks of rainbow trout, *Oncorhynchus mykiss* (Walblum), fry syndrome in England. *J Fish Dis*., vol. 14, no. 6, p. 677-682.

AVENDAÑO-HERRERA, R., MAGARIÑOS, B. LÓPEZ-ROMALDE, S., ROMALDE, JL. and TORANZO, AE., 2004. Phenotipic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains from marine fishes. *Dis. Aquat. Org*., vol. 58, no.1, p. 1-8.

BAXA, DV., KAWAI, K. and KUSUDA R., 1988. In vitro and in vivo activities of *Flexibacter maritimus* toxins. *Reports of USA Marine Biology Institute. Kochi University,* vol. 10, no. 1, p. 1-8.

-, 1986. Characteristics of glinding bacteria isolated from disease cultured flounder, *Paralichthys olivaceus. Fish Pathol.*, vol. 21, no. 4, p. 251-258.

BERNARDET, JF., CAMPBELL, AC. and BUSWELL, JA., 1990. *Flexibacter maritimus* is the agent of "Black patch necrosis" in Dover sole in Scotland. *Dis. Aquat. Org.*, vol. 8, p. 233-237.

BERNARDET, JF. and GRIMONT, P., 1989. Desoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus*. *International Journal of Systematic Bacteriology*, vol. 39, no. 3, p. 346-354.

BERTOLINI, JM., WAKABAYASHI H, WVG., WHIPPLE, MJ. and ROHVEC, JS., 1994. Electrophoretic detection of proteases from selected strains of *Flexibacter psychrophilus* and assessment of their variability. *J Aquat Anim Health,* vol. 6, no. 3, p. 224-233.

BOWER, SM., MACGLADDERY, SE., PRICE, IM., 1994. *Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish: Filamentous Bacterial Diasease of Shrimp and Prawns.* Available in: http://www.sci.pac.dfo mpo.gc.ca/shelldis/pages/filbdsp_e.htm. [Accessed in: July 11, 2005].

CHEN ME. and HENRY-FORD D., 1995. Isolation and Characterization of *Flexibacter maritimus* from Marine Fishes of California. *J. Aquat Anim Health,* vol. 7, no. 4, p. 318-326.

HANSON, LA. and GRIZZLE, JM., 1985. Nitrite induced predisposition of channel catfish to bacterial disease. *Progres. Fish Cult.*, vol. 47 no. 2, p. 98-101.

KAMPFER, P., 1997. Detection and cultivation of filamentous bacteria from activated sludge. *Fems Micriobiol. Ecology*, vol. 23, no. 3, p. 169-181.

KITATSUJI, K., MIYATA, H. and FUKASE, T., 1996. Lysis of filamentous bacteria by surfactants. *Water Sci. Tec.*, vol. 34, no. 5, p. 145-153.

LEWIN, RA., LOUNDSBERY, DM., 1969. Isolation, cultivation and characterization of Flexibacteria. *J. Gen. Microbiol.*, vol. 58, p. 145-170.

LIGHTNER, DV., 1988. Filamentous bacterial disease of penaeid shrimp. In SINDERMANN, CJ. and LIGHTNER, DV. (Eds.). *Disease Diagnosis and Control in North American Marine Aquaculture.* Developments in Aquaculture and Fisheries Science 17. Elsevier, Amsterdam, p. 52-57.

LIGHTNER, DV., 1996. Disease of cultured penaeid shrimp. In MCVEY, JP. (Ed), *Handbook of Mariculture*. *Crustacean Aquaculture*, 2nd Ed. CRC Press, Boca Raton, Florida, USA. p. 393-486.

LUMSDEN, JS., OSTLAND, VE. and FERGUSON, HW., 1996. Necrotic myositis in cage cultured rainbow trout, *Oncorhynchus mykiss* (Walblum), caused by *Flexibacter psychrophilus. J Fish Dis.*, vol. 19, no. 2, p. 113-119.

OSTLAND, VE., MCGROGAN, DG. and FERGUSON, HW., 1997.Cephalic osteochondritis and necrotic scleritis in intensively reared salmonids associated with *Flexibacter psychrophilus. J Fish Dis.*, vol. 20, no. 6, p. 443-451.

POWELL, M., CARSON, J. and VAN GELDEREN, R., 2004. Experimental induction of gill disease in Atlantic salmon *Salmo salar* smolts with *Tenacibaculum maritimum. Dis. Aquat. Org.*, vol. 61 no. 3, p. 179-185.

SKJERMO, J. AND VADSTEIN, O., 1999. Techniques for microbiological control in the intensive rearing of marine larvae. *Aquaculture*, vol. 177, no. 4, p. 333-343.

SOLTANI, M., SHANKER, S., MUNDAY, BL.. 1995. Chemotherapy of Cytophaga/Flexibacter-like bacteria (CFLB) infections in fish: studies validating clinical efficacies of selected antimicrobials. *J Fish Dis*., vol. 18, no. 6, p. 555-565.

WAKABAYASHI, H., HIKIDA, M. and MASUMURA, K. 1984. *Flexibacter* infection in cultured marine fish in Japan. *Helgolander Meeresunter-suchungen,* vol. 37, no. 4, p. 587-593.

WAKABAYASHI, H., HIKIDA, M. and MASUMURA, K., 1986. *Flexibacter maririmus* sp. nov., a pathogen of marine fishes. *International Journal of Systematic Bacteriology*, vol. 36, no. 3, p. 396-398.

YASUDA, K. and KITAO, T., 1983. Bacterial flora in the digestive tract on praws, *Penaeus japonicus* Bate. *Aquaculture*, vol. 19, no. 3, p. 229-234.