[4] Diseases Caused By Bacterial Pathogens in Saltwater

6. TENACIBACULOSIS

Hisatsugu Wakabayashi

6.1. Synopsis

The genus *Tenacibaculum*, within family Flavobacteriaceae, consist of Gram negative, long thin bacteria which are not flagellated and are motile by gliding when in contact with a solid surface. They grow well on low nutrient media containing sea water producing pale yellow rhizoid colonies. *Tenacibaculum maritimum* is one of the most threatening bacterial pathogens for a wide range of cultured marine fish species in the world. *Tenacibaculum discolor* and *Tenacibaculum soleae* are the isolates from diseased cultured sole (*Solea senegalensis)* in Spain. The diseased fish are similar in appearance to those with columnaris disease in freshwater fish. *Tenacibaculum ovoliticum* is an opportunistic pathogen for Atlantic halibut (*Hippoglossus hippoglossus*) eggs and larvae in Norway.

6.2. Introduction

Outbreaks of columnaris-like gliding bacterial diseases were reported in salmonid fishes cultured in sea water in the North America, but the causative agent was not able to be specified (Borg, 1960; Wood, 1974; Anderson and Conroy, 1969; Sawyer,1976). Masumura and Wakabayashi (1977) described outbreaks of a gliding bacterial disease in hatchery born red sea bream (*Pagrus major)* and black sea bream (*Acanthopagrus shlegeli)* fry reared in marine net-cages in Japan. The diseased fish were similar in appearance to those with columnaris disease and large numbers of gliding bacteria were observed in scrapings from lesions. An organism was isolated on Cytophaga agar (Anacker and Ordal 1959) prepared with seawater, and the disease condition was produced by experimental infections. The causative bacteria obligatorily required sea water for growth and this could not be replaced by NaCl alone (Hikida *et al*. 1979). Wakabayashi *et al*. (1986) proposed the name *Flexibacter maritimus* sp nov. for the organism. Subsequently *F. maritimus* infection was reported in Japanese flounder (*Paralichthys olivaceus*) reared in marine hatcheries in Japan (Baxa et al. 1986, 1987).

An investigation carried out by Bernardet and Grimont (1989) revealed that a strain of a '*Flexibacter columnaris*-like' bacterium depositied in the National Collection of Marine Bacteria (strain NCMB 2158) was synonymous with *F. maritimus*. This strain was originally reported as the etiological agent of 'black patch necrosis' (BPN) in Dover sole (*Solea solea*) by Campbell and Buswell (1982). Then, *F. maritimus* infection was reported by various marine fishes cultured in France and Spain (Alsina and Blanch, 1993; Pazos et al, 1993; Bernardet et al., 1994).

Chen and Henry-Ford (1995) isolated *F. maritimus* from the gill and skin lesions of chinook salmon (*Oncorhynchus tschawytscha*) and white sea bass (*Atractoscion nobilis*) reared in marine net-pens along the southern California coast. They also recovered *F. maritimus* in lesions of northern anchovy (*Engraulis mordax*) and Pacific sardine (*Sardinops sagax*) which were used as live bait. Handlinger et al. (1997) reported *F. maritimus* infection of cultured marine fishes, such as striped trumpeter (*Latris lineata*), salmonid fishes and greenback flounder (*Rhombosolea tapiria*) in Tasmania, Australia.

Kent et al. (1988) reported pathological changes and mortality associated with a *Cytophaga* infection in Atlantic salmon (*Salmo salar*) smolts maintained in net pens along the coast of Washington State, USA. Gliding bacteria were isolated on the seawater Cytophaga agar (SCA) from lesions, and were identified as a *Cytohpaga* sp. The organism was a marine bacterium requiring at least 10% seawater for growth, but serologically distinct from *F. maritimus*. Two new species of the genus *Tenacibaculum*, i.e. *Tenacibaculum discolar* and *Tenacibaculum soleae* were isolated from diseased cultured sole (*Solea senegalensis*) which showed the typical signs observed in fish affected by *T. maritimum* (Pineiro-Vidal *et al*., 2008a; Pineiro-Vidal *et al*., 2008b). A psychrotrophic *T. ovolyticum* (syn. *Flexibacter ovolyticus*) was isolated for the adherent bacterial epiflora of Atlantic halibut (*Hippoglossus hippoglossus)* eggs and was shown to be an opportunistic pathogen for halibut eggs and larvae (Hansen et al., 1992).

6.3. Disease Agent

The gliding bacteria isolated from diseased sea breams in Japan was thought as a new species of *Flexibacter*, for which the name of *Flexibacter maninus* was mooted (Hikida et al., 1979). Subsequently, Wakabayashi et al., 1986) formally proposed the name of *Flexibacter maritimus* to accommodate the pathogens. Although renaming the organism *Cyophaga marina* was proposed by Reichenbach (1989), it was judged that *F. maritimus* was a priority species name (Holmes, 1992). Suzuki et al. (2001) pointed out that *F. maritimus* and *F. ovolyticus* were genetically misclassified bacteria, because these species were distantly related to *Flexibacter flexilis*, the type species of the genus *Flexibacter*. They proposed the reclassification of *F. maritimus* and *F. ovolyticus* into *Tenacibaculum* gen. nov., as *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov.

T. maritimum has an absolute requirement for sea water. No growth occurs on cytophaga agar (Anacker and Ordal, 1959) with the addition of NaCl instead of seawater. At least 30% seawater is required. The bacterium requires KCl as well as NaCl for growth. Ca^{++} enhances growth while SO^{++} is slightly inhibitory (Hikida et al., 1979). In cultivation of *T. maritimum*, seawater cytophaga agar (Masumura and Wakabayashi, and 1977), TYC (Hikida et al., 1979), Marine 2216E (Difco) (Bernardet et a. 1994), FMM (Pazos et al., 1996) and SFM (Bullock et al., 1986) have been used. Colonies on seawater cytophaga agar are pale yellow, flat and thin with uneven edges and adherent to the agar (Figure 6.1). The pigment is not flexirubin-type. In un-agitated liquid medium, surface growth is in the form of a pellicle. The growth occurs from 14.6 to 34.3^{o}C. Bacterial cells from fresh culture are Gram-negative, flexible slender rods (0.3-0.5 x 2-30 μm). As the cultures age, however, the cells tend to become somewhat shorter and produce round bodies. These spherical cells are not capable of germinating in fresh medium. The organisms have no flagella but exhibit gliding motility on a wet surface. Although columnar formation is not so obvious as in *F. columnare*, *T. maritimum* also gathers into masses on the periphery of isolated tissues on wet mount preparations (Figure 6.2). *T. maritimum* produces catalase, cytochrome oxidase and ammonium and hydrolyses casein, gelatin, tributyrin and tyrosin. It does not produce hydrogen sulphate or indole. Nitrogenous compounds such as tryptone, yeast extract and casamino acid are utilized as sources of carbon and nitrogen for growth. Agar, cellulose, chitin, starch and aesculin are not degraded. Nitrate is reduced to nitrite. Acid is not produced from glucose, galactose, fructose, mannose,

lactose, sucrose, sorbose, maltose, cellobiose, trehalose, xylose, rhamnose, raffinose, dextrin, glycogen, inulin, glycerol, adonitol, sorbitol, inositol or salicin. The G+C ratio of the DNA is a 31.3-32.5mol % (Hikida et al. 1979; Wakabayashi et al. 1986).

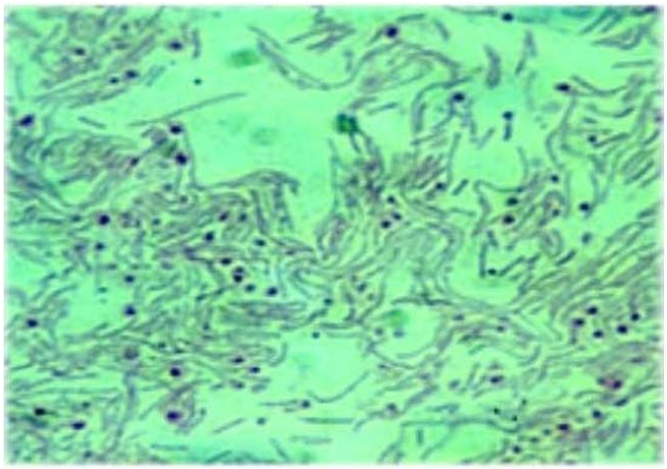

Figure 6.1. *Tenacibaculum maritimum* cells pure-cultured on Seawater Cytophaga broth, showing a mixture of slender rods and round bodies.

T. ovolyticum is Gram-negative, long, slender rods (0.4 x 2-20 μm) which occasionally grow to filaments that are 70 to 100μm long. Colonies are Kovacs oxidase positive and pale yellow. Microcysts are not formed. The cells exhibit gliding motility, do not adsorb Congo red, and do not possess a flexirubin type of pigment. It is strictly aerobic and does not produce acid from carbohydrates. Gelatin, tyrosine, DNA, and Tween 80 are degraded, but starch, cellulose, and chitin are not degraded. It possesses catalase and nitrate reductase activities. H_2S is not produced, and 50% seawater is required for growth. *T. ovolyticum* grows at 4°C, but not at 30°C. The G＋C ratio of the DNA is a 30.3-32.0 mol% (Hansen et al., 1992)

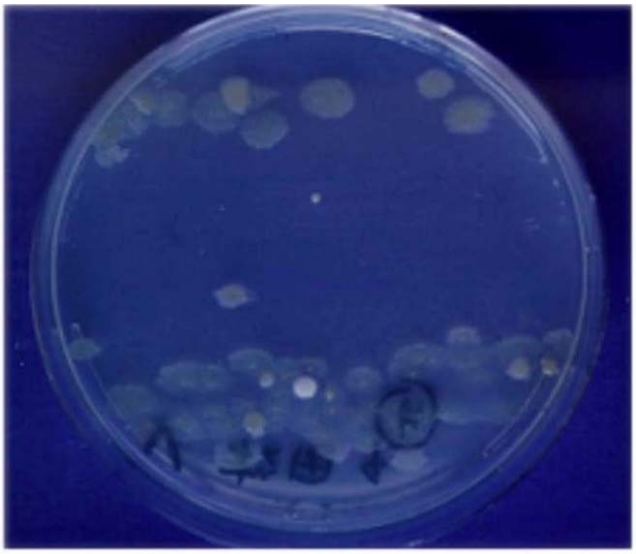

Figure 6.2. Colonies of *Tenacibaculum maritimum* isolated from a lesion tissue on Seawater Cytophaga agar, including a few other bacterial colonies.

Agglutination tests using polyclonal antisera showed cross-reaction for all strains regardless of host species and serum employed, and the heterologous titers were similar to homologous ones (Wakabayashi et al., 1984; Ostland et al., 1999; Avendano et al., 2004). Immunodifusion and western immunoblot analysis demonstrated distinct antigenic differences among the strains (Ostland et al., 1999).

In accordance with the results obtained with absorbed antisera, the strains were provisionally divided into 2 serological groups (Avendano et al., 2004). Immunoblot analysis of the LPS clearly revealed serotypes O1 and O2 that were distinguishable without the use of absorbed antiserum (Avendano et al., 2004) Avendano et al. (2005) reported the existence of a new serotype, which was proposed as serotype O3. These serotypes mainly associated with the host species (Avendano et al., 2005)

The ability of *T. maritimum* to produce disease in various fish species was examined by many authors and mortalities among experimental fish varied widely depending on the method of infection. The disease was not induced by intramascular or intraperitoneal injection of the pathogens in experimentally infected red and black sea bream, sea bass or turbot (Wakabayashi et al., 1984; Alsina & Blanch, 1993; Pepin & Emery, 1993; Bernardet et al., 1994; Avendano et al., 2006). Fatal infections occurred most frequently when fish were exposed to topical application of the culture on the surface of the mouth or tail (Wakabayashi et al., 1984). However, Campbell & Buswell (1982) challenged two group of 10 Dover sole with a bacterial suspension. The group challenged by scarification showed no signs of the disease, but the group injected subdermally had a 30% mortality after 48h. Bath challenge was not a reliable method of inducing the disease in sea bass unless the skin is previously scarified (Wakabayashi et al., 1984; Baxa et al, 1987; Bernardet et al., 1994) or the bacterial strain is first passaged twice in Atlantic salmon (Handlinger et al., 1997). Avendano et al., (2006) demonstrated that, using prolonged immersion of turbot for 18h with the pathogen at 18 to20^{o}C, the disease could be easily reproduced with the fish showing the classical signs of the disease.

6.4. Diagnostic Methods

In the case of the fry of red sea bream or black sea bream, the clinical signs of *T. maritimum* infection are loss of appetite, lethargy and darkening of the body surface. The diseased fish swim abnormally near the edge of net cage. They have eroded mouths, frayed fines and tail rot (Figure 6.3). In the lesions large numbers of long, slender rod-shaped bacteria are observed and give the infected tissue a pale yellow appearance. In older fish the lesions occur initially as grey-white cutaneous foci on the fins, head and trunk. On the skin, the lesions become eroded and shallow ulcers are produced (Masumura and Wakabayashi 1977, Wakabayashi et al. 1984). McVicar and White (1979) described the clinical signs of ‘black patch necrosis’ in Dover sole as slight blistered of the skin surface or darkening of tissue between caudal and marginal fin rays followed by extensive darkening of the area, loss of the epithelial surface and hemorrhage in exposed dermal tissues. Similar clinical signs of *Tenatibaculum* infection were reported on many kinds of fishes, i.e. Japanese flounder (Baxa et al. 1986), Atlantic salmon (Kent et al. 1988; Handlinger et. al. 1997, Ostland et al. 1999, Olsen et al. 2011), turbot (Alicina and Blanch 1993), sea bass (Bernardet et al., 1994), white sea bass (Chen et al., 1995), Pacific sardine (Chen et al., 1995), Chnook salmon (Chen et al., 1995),

rainbow trout (Handlinger et al. 1997), green flounder (Handlinger et al., 1997), striped trumpeter (Handlinger et al. 1997), wedge sole (Lopez et al., 2009), Senegal sole (Vilar et al. 2012), and Asian sea bass (Gibson-Kueh et al., 2012). The presumptive diagnosis is based on the clinical signs of the diseased fish, particularly gross external lesions, as well as in the microscopic examination of the lesions revealing long, thin, rod-shaped bacteria which show gliding motility in wet mounts.

Figure 6.3. Tail rot caused by *Tenacibaculum maritimum* in juvenile black sea bream (upper) and red seabream (lower). Eroded mouth and frayed fins are also observed.

Figure 6.4. Cells of *Tenacibaculum maritimum* swarming on the margin of eroded fin tissues.

Diagnosis can be confirmed by the isolation of bacterial colonies on an appropriate medium such as seawater cytophaga agar, TYC, Marine 2216E, FMM, or SFM, followed by morphological and biochemical characterization. This traditional method is time consuming and somewhat difficult to distinguish *T. maritimum* from other phenotypically similar species. In addition, due to the slow growth characteristic of *T. maritimum*, the mixed other bacterial species sometimes overgrow the *T. maritimum* colonies. The development of polymerase chain reaction (PCR)-based methods offer the possibility of

more rapid and accurate identification of the pathogen from plate cultures as well as from tissues. Toyama et al. (1996) designed a pair of primers MAR1 (5'-AATGGCATCGTTTTAAA-3') and MAR2 (5'-CGCTCTCTGTTGCCAGA-3') for the detection of *T. maritimum* using 16S rRNA gene as target. Bader and Shotts (1998) also selected a pair of *T. maritimum* species-specific PCR primers Mar1 (5'-TGTAGCTTGCTACAGATGA-3') and Mar2 (5'-AAATACCTACTCGTAGGTACG-3') targeted 16S rRNA gene. In order to increase the sensitivity, two nested PCR protocols for detection of *T. maritimum* based on MAR1-MAR2 and Mar1-Mar2 were developed (Avendano-Herrera et al., 2004a,c; Cepeda et al. 2003). PCR-enzyme-linked immnosorbent assay (PCR-ELISA)(Wilson et al., 2002), reverse transcriptase polymerase chain reaction-enzyme hybridization assay (RT-PCR-EHA)(Wilson and Carson 2003) and DNA microarray probe (Warsen et al. 2004) are also available for the direct detection of *T. maritimum* from pure culture.

6.5. Control

T. malitimum is sensitive to various antibiotics *in vitro* studies (Alicna and Blanch 1993, Pazos et al. 1993, Chen et al., 1995, Soltani et al. 1995. Avendano-Herrera et al. 2005) and therefore any approved drug can be used. However, field results are not always satisfactory even if the isolated bacteria are highly sensitive to the drug (McVicar and White, 1979, Handlinger et al 1997, Sepeda and Santos 2002).

As with other infections, avoiding overcrowding and overfeeding is recommended, particularly during the expected time of outbreak (McVicar and White 1982, Wakabayashi 1993, Soltani et al 1996). McVicar and White (1979, 1981) demonstrated that the addition of a sand substrate to tanks would rapidly cure the disease in juvenile Dover sole and remarkably improve hatchery survivals.

Development of a vaccine has been pursued in Spain and Australia (Tranzo et al. 2005, van Gelderen et al. 2009), but no practical, commercially available vaccine has been marketed.

6.6. Recent Topics (Jellyfish as a vector of *T. maritimum*)

Ferguson et al. (2010) demonstrated that *T. maritimum* was present on the mouth of jellyfish, such as *Phialella quadrata* and that their DNA sequences were almost identical to those of *T. maritimum* present on the gill of farmed Atlantic salmon in Scotland. They thought that the initial damage to gills, likely produced by nematocyst-derived toxins from the jellyfish, was compounded by secondary bacterial infection with *T. maritimum*, and that *P. quadrata* was probably acting as a vector for the pathogen. By the use of real-time PCR, *T. maritimum* DNA was detected at low level in four of 26 jellyfish, three *P. quadrata* and one *Muggiaea atlantica*, which were collected in summer 2010 from seawater locations around Ireland (Fringuelli et al. 2012). Several interesting questions were raised (Ferguson et al. 2010). What is the relationship between the bacteria and the jellyfish? Are they a pathogen of the jellyfish, or are they present in a symbiotic or commensal capacity?