

## REVIEW

# Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review

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**ABSTRACT:** *Tenacibaculum maritimum* is the aetiological agent of an ulcerative disease known as tenacibaculosis, which affects a large number of marine fish species in the world and is of considerable economic significance to aquaculture producers. Problems associated with epizootics include high mortality rates, increased susceptibility to other pathogens, high labour costs of treatment and enormous expenditures on chemotherapy. In the present article we review current knowledge on this bacterial pathogen, focusing on important aspects such as the phenotypic, serologic and genetic characterization of the bacterium, its geographical distribution and the host species affected. The epizootiology of the disease, the routes of transmission and the putative reservoirs of *T. maritimum* are also discussed. We include a summary of molecular diagnostic procedures, the current status of prevention and control strategies, the main virulence mechanisms of the pathogen, and we attempt to highlight fruitful areas for continued research.

**KEY WORDS:** *Tenacibaculum maritimum* · Tenacibaculosis · Review · Pathogenicity · Diagnosis · Control

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## INTRODUCTION

*Tenacibaculum maritimum*, a Gram-negative and filamentous bacterium, has been described as the etiological agent of tenacibaculosis in marine fish. The pathology of the disease caused by this marine organism has mainly been associated with characteristic gross lesions on the body surface of fish such as ulcers, necrosis, eroded mouth, frayed fins and tail rots, and sometimes necrosis on the gills and eyes (McVicar & White 1979, Campbell & Buswell 1982, Wakabayashi et al. 1984, Baxa et al. 1986, Devesa et al. 1989, Alsina & Blanch 1993, Chen et al. 1995, Handlinger et al. 1997, Ostland et al. 1999, Cepeda & Santos 2002). However, due to some variation in external pathological signs of the disease, depending on the species and age of fish, some authors have used different names to designate this ulcerative condition (see reviews by Bernardet 1998 and Santos et al. 1999). For example, the names salt water columnaris

disease, gliding bacterial disease of sea fish, bacterial stomatitis, eroded mouth syndrome and black patch necrosis have all been used. To avoid confusion with other fish diseases, this review will use the name tenacibaculosis.

To date, tenacibaculosis is one of the most threatening bacterial infections limiting the culture of many species of commercial value in distinct geographical areas of the world (see review by Toranzo et al. 2005). Therefore, the aim of this article is to compile the current state of knowledge of the disease caused by *Tenacibaculum maritimum*, focusing on important aspects such as a phenotypic, serological and genetic characterization of the bacterium, and a description of the pathogen's geographical distribution and the host species affected. In addition, methods to diagnose together with strategies to prevent the disease and its main virulence mechanisms have also been addressed. Our hope is that this review will highlight important directions for future research on this fish pathogen.

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## TAXONOMY

The taxonomy of *Tenacibaculum maritimum* was a matter of controversy and confusion for decades, and it has only recently been clarified by Suzuki et al. (2001). Masumura & Wakabayashi (1977) isolated a gliding bacterium that had caused mass mortalities among certain cultured marine fish. These strains were characterized by Hikida et al. (1979), who announced their intention to make a separate formal proposal of the name *Flexibacter marinus*. Since the epithet *marinus* had already been used in the name *Vibrio marinus*, the authors changed their mind on the use of this epithet, and the eventual formal proposal was a taxon called *Flexibacter maritimum* (Wakabayashi et al. 1986, Holmes 1992). Reichenbach (1989) listed the pathogen as *Cytophaga marina*, but the priority of the name *Flexibacter maritimum* was later recognized (Holmes 1992). These results were confirmed by Bernardet & Grimont

(1989), who also validated the name *Flexibacter maritimum* based on DNA-DNA hybridisation methods.

However, the resolution of phenotypic characterization and 16S ribosomal RNA (rRNA) sequence analysis is insufficient to distinguish closely related organisms. Thus, Suzuki et al. (2001), based on the nucleotide sequence of the *gyrB*, proposed that *Flexibacter maritimum* should be transferred to the new genus *Tenacibaculum*, in which 7 members are currently included. Table 1 shows the main differential characteristics of the described species of the genus *Tenacibaculum*.

## GEOGRAPHICAL DISTRIBUTION AND HOST SPECIES

The geographical distribution of *Tenacibaculum maritimum* in wild and farmed fish is shown in Table 2.

Table 1. Differential phenotypic characteristics of *Tenacibaculum* species. Data are from Wakabayashi et al. (1986), Hansen et al. (1992), Suzuki et al. (2001), Frette et al. (2004) and Yoon et al. (2005). +: positive; -: negative; W: weakly positive; V: variable reaction; ND: not determined; NG: no growth in the presence of NaCl only. All species are Gram-negative and rod-shaped. All species are positive for catalase, oxidase and degradation of casein

Characteristic	<i>T. maritimum</i>	<i>T. ovolyticum</i>	<i>T. mesophilum</i>	<i>T. amilyticum</i>	<i>T. skagerrakense</i>	<i>T. lutimaris</i>
Origin	Diseased red sea breem fingerling, Japan	Halibut egg, Norway	Sponge and macroalgae, Japan	Macroalgae, Japan	Pelagic, Denmark	Tidal flat, Korea
Cells size (µm)	2–30 × 0.5	2–20 × 0.5	1.5–10 × 0.5	2–4 × 0.4	2–15 × 0.5	2–10 × 0.5
Colony morphology						
Shape	Uneven edge	Regular edge	Circular or irregular, spreading edge	Circular, spreading edge	Circular, spreading edge	Irregular, spreading edge
Colour	Pale yellow	Pale yellow	Yellow	Yellow	Bright yellow	Pale yellow
Temperature range (°C)	15–34	4–25	15–40	20–35	10–40	10–39
Optimal temperature (°C)	30	ND	28–35	27–30	25–37	30–37
Salinity range						
Seawater (%) <sup>a</sup>	30–100	70–100	10–100	50–100	25–150	ND
NaCl (%) <sup>b</sup>	NG	NG	1–7	3 (W)	NG	2–3
pH range	5.9–8.6	5.9–8.6	5.3–9.0	5.3–8.3	6.0–9.0	7.0–8.0
Growth on:						
Casamino acids	+	V	+	+	ND	+
Sucrose	–	–	–	–	+	–
D-ribose	–	–	–	–	ND	–
DL-aspartate	–	–	+	–	+	–
L-proline	–	–	+	+	+	–
L-glutamate	W	–	+	+	+	–
L-leucine	–	–	–	–	W	–
Degradation of:						
Starch	–	–	–	+	+	–
Gelatin	+	+	+	+	ND	+
Tween 80	+	+	+	+	–	–
Nitrate reduction	+	+	–	W	+	V (–)
G + C content (mol%)	31.3–32.5	30.3–32	31.6–32	30.9	35.2	32.3–32.8

<sup>a</sup>100 = full-strength seawater  
<sup>b</sup>Percentage of NaCl in the medium

Table 2. *Tenacibaculum maritimum*. Geographic origin and host species identified to date

Host species	Country	Source
Asia		
Black sea bream <i>Acanthopagrus schlegeli</i>	Japan	Masumura & Wakabayashi (1977), Wakabayashi et al. (1984, 1986)
Red sea bream <i>Pagrus major</i>	Japan	Masumura & Wakabayashi (1977), Wakabayashi et al. (1984, 1986)
Japanese flounder <i>Paralichthys olivaceous</i>	Japan	Baxa et al. (1986)
Yellowtail <i>Seriola quinqueradiata</i>	Japan	Baxa et al. (1988b,c)
Rock bream <i>Oplegnathus fasciatus</i>	Japan	Wakabayashi et al. (1986)
Europe		
Dover sole <i>Solea solea</i>	UK	McVicar & White (1979, 1982), Campbell & Buswell (1982), Bernardet et al. (1990)
Sole <i>Solea senegalensis</i>	Portugal	Cepeda & Santos (2002), Avenidaño-Herrera et al. (2004b, 2005b)
	Spain	Avenidaño-Herrera et al. (2004b, 2005b)
Sea bass <i>Dicentrarchus labrax</i>	France	Pépin & Emery (1993), Bernardet et al. (1994)
	Malta	Bernardet (1998)
	Italy	Salati et al. (2005)
Turbot <i>Scophthalmus maximus</i>	Spain	Devesa et al. (1989), Alsina & Blanch (1993), Pazos et al. 1993, Avenidaño-Herrera et al. (2004a,b, 2005b)
Atlantic salmon <i>Salmo salar</i>	Spain	Pazos et al. (1993)
Gilthead seabream <i>Sparus aurata</i>	Spain	Avenidaño-Herrera et al. (2004a,b)
Tub gurnard <i>Chelidonichthys lucerna</i>	Italy	G. Magi (unpubl. data)
Oceania		
Atlantic salmon <i>Salmo salar</i>	Australia	Soltani & Burke (1994), Handlinger et al. (1997), Powell et al. (2004)
Rainbow trout <i>Oncorhynchus mykiss</i>	Australia	Soltani et al. (1996), Handlinger et al. (1997)
Striped trumpeter <i>Latris lineata</i>	Australia	Handlinger et al. (1997)
Greenback flounder <i>Rhombosolea tapiriña</i>	Australia	Soltani et al. (1996), Handlinger et al. (1997)
Yellow-eye mullet <i>Aldrichetta forsteri</i>	Australia	Handlinger et al. (1997)
Black bream <i>Acanthopagrus butcheri</i>	Australia	Handlinger et al. (1997)
America		
White sea bass <i>Atractoscion nobilis</i>	USA	Chen et al. (1995)
Pacific sardine <i>Sardinops sagax</i>	USA	Chen et al. (1995)
Northern anchovy <i>Engraulis mordax</i>	USA	Chen et al. (1995)
Chinook salmon <i>Oncorhynchus tshawytscha</i>	USA	Chen et al. (1995)
Atlantic salmon <i>Salmo salar</i>	Canada	Ostland et al. (1999)

Marine tenacibaculosis infection was first described by Masumura & Wakabayashi (1977) as the cause of mortalities in red *Pagrus major* and black sea bream *Acanthopagrus schlegeli*, when a massive epizootic occurred in a hatchery in Hiroshima Prefecture (Japan). A few years later, the disease spread to other important cultured fish species in Japan such as Japanese flounder *Paralichthys olivaceous* and yellowtail *Seriola quinqueradiata*, among others (Baxa et al. 1986, Wakabayashi et al. 1986).

Despite the fact that tenacibaculosis has long been recognized as a disease of fish in Japan, the infection was not considered of economical importance until serious mortalities affecting Dover sole *Solea solea* in Scotland were reported by McVicar & White (1979, 1982). The disease was later described as affecting sea bass *Dicentrarchus labrax* farmed on the Mediterranean coast of France (Pépin & Emery 1993, Bernardet et al. 1994). Since 1990, as the farming of

fish became a steadily growing industry, outbreaks of tenacibaculosis have also occurred in Spain and Portugal, mainly in turbot *Scophthalmus maximus* populations, Atlantic salmon *Salmo salar* and, lately, in sole *Solea senegalensis* and *S. solea* and gilthead seabream *Sparus aurata* (Devesa et al. 1989, Alsina & Blanch 1993, Pazos et al. 1993, Cepeda & Santos 2002, Avenidaño-Herrera et al. 2004a,b, 2005b). Recently, *Tenacibaculum maritimum* has been isolated in Italy from sea bass (Salati et al. 2005) and tub gurnard *Chelidonichthys lucerna* (G. Magi unpubl. data).

In the southern hemisphere, *Tenacibaculum maritimum* has been identified in Australia as a pathogen of sea-caged Atlantic salmon and rainbow trout *Oncorhynchus mykiss*, captured striped trumpeter *Latris lineata*, greenback flounder *Rhombosolea tapiriña*, yellow-eye mullet *Aldrichetta forsteri* and black bream *Acanthopagrus butcheri* (Soltani & Burke 1994, Handlinger et al. 1997). On the American continent, the

microorganism has also been isolated from captured white sea bass *Atractoscion nobilis*, Pacific sardine *Sardinops sagax* and northern anchovy *Engraulis mordax* as well as Chinook salmon *Oncorhynchus tshawytscha* reared in marine net-pens along the southern Californian coast (Chen et al. 1995). Since then, outbreaks of tenacibaculosis have been reported in other geographical areas of North America, affecting Atlantic salmon smolts from British Columbia, Canada (Ostland et al. 1999). *T. maritimum* is suspected to be responsible for fish mortalities in Chile (Bernardet 1998), but the isolates have not been fully identified and, to our knowledge, no confirmation has as yet been published.

We believe that *Tenacibaculum maritimum* affects other host populations, in either cultured and/or wild fish; however, the failure to recover this pathogen from field samples, probably in part owing to its demanding growth requirements, makes the diagnosis of tenacibaculosis as well as the correct identification of the etiological agent difficult.

#### ISOLATION AND PHENOTYPIC CHARACTERIZATION

The isolation of *Tenacibaculum maritimum* using classical agar cultivation is difficult due to the slow growth of the bacterial cells and the overgrowth and/or inhibition produced by several other bacterial species present within the lesions (Pazos et al. 1996, Avendaño-Herrera et al. 2004a). The pathogen is an obligate marine microorganism and does not grow on media prepared only with NaCl; it must be cultured on non-selective oligotrophic media that contain at least 30% seawater (Wakabayashi et al. 1986).

Since the first reports of tenacibaculosis, Anacker and Ordal agar (AOA) (Anacker & Ordal 1959) or several modifications of this medium, prepared in 70% seawater, have been advocated for the isolation of seawater-dependent slow-growing *Tenacibaculum maritimum* from infected fish (Hikida et al. 1979, Wakabayashi et al. 1984, 1986, Bullock et al. 1986, Handlinger et al. 1997). Marine agar (Campbell & Buswell 1982, Alsina & Blanch 1993) and Hsu-Shotts media supplemented with antibiotics (Chen et al. 1995) have also been devised for the recovery and isolation of the pathogen. Other basal media, i.e. tryptone casamino acids yeast extract (Wakabayashi et al. 1984, 1986, Ostland et al. 1999), tryptone yeast extract salts (Toyama et al. 1996), tryptone yeast (Bader & Shotts 1998) and 1/5 LBM (Marine Luria Broth) (Suzuki et al. 2001), have been described for laboratory culture and study of some physiological characteristic of *T. maritimum*, but they are not recommended for its isolation

from environmental samples. In fact, although all these media support the growth of the *T. maritimum* strains, another medium named *Flexibacter maritimus* medium (FMM) (Pazos et al. 1996) has been proposed as the most appropriate for the successful isolation of this species from fish tissue. *T. maritimum* seems to grow better on this medium in comparison to heterotrophic halophilic bacteria such as *Vibrio*, *Pseudomonas* and *Alteromonas* spp., which are usually present in skin samples. This medium, as well as FMM prepared with commercial sea salts instead of seawater, has also been recommended for use in the routine drug susceptibility testing of this demanding pathogen (Avendaño-Herrera et al. 2005a). The incubation on most media is usually carried out at 20 to 25°C for 48 to 72 h. In addition, it is important to note that only on FMM and AOA do the colonies exhibit the typical features described for this species—flat, pale-yellow with uneven edges and strongly adherent to the medium—whereas on marine agar, colonies are round and show yellow pigment (Pazos et al. 1996).

The morphological, physiological and biochemical characteristics useful in the identification of *Tenacibaculum maritimum* have been detailed by several authors (Hikida et al. 1979, Baxa et al. 1986, Wakabayashi et al. 1986, Bernardet & Grimont 1989, Bernardet et al. 1990, 1994, Alsina & Blanch 1993, Pazos et al. 1993, Soltani & Burke 1994, Chen et al. 1995, Ostland et al. 1999, Suzuki et al. 2001, Avendaño-Herrera et al. 2004b). These phenotypic results indicate that *T. maritimum* constitutes an homogeneous species, and they can be used for the identification of the pathogen. *T. maritimum* is a filamentous bacterium 0.5 µm wide by 2 to 30 µm long; however, occasionally cells up to 100 µm in length can be observed. In older liquid and solid cultures, cells appear shorter and tend to become spherical (approximately 0.5 µm). Spheroplast-like forms are rarely observed even after prolonged incubation of the *T. maritimum* cells. Gliding motility on wet surfaces is a general feature of all isolates. The bacterium is mesophilic and can grow at temperatures from 15 to 34°C, with an optimum growth temperature of 30°C. On solid media, *T. maritimum* colonies absorb Congo red but the cells do not contain cell wall-associated flexirubin-type pigment. Different types of colonies, varying in their rhizoid aspect and adherence, may coexist on the same agar plate (Sorongon et al. 1991).

*Tenacibaculum maritimum* produces enzymes that degrade casein, tyrosine and tributyrin, but it does not hydrolyse agar, carboxymethyl cellulose, cellulose, starch, esculin, or chitin. Variable results have been reported for gelatin, hydrogen sulfide and nitrate reactions. As suggested by Suzuki et al. (2001), the employment of different basal media could account for this

variability. Catalase and oxidase are positive in all strains. Regarding the use of carbohydrates, this pathogen is not able to degrade most of the simple and more complex carbohydrates. In addition, growth occurs on tryptone, casamino acids and yeast extract as the sole carbon and nitrogen source.

## SEROLOGICAL AND GENETIC STUDIES

The first serological studies described by Wakabayashi et al. (1984) and Pazos et al. (1993), based on slide agglutination assays, reported antigenic homogeneity of *Tenacibaculum maritimum*, regardless of their origin and source of isolation. Further studies by Pazos (1997) and Ostland et al. (1999) demonstrated antigenic differences among *T. maritimum* isolates, suggesting that this microorganism may not be as homogeneous as previously thought. However, no agreement was reached among the serological groups established by these authors, perhaps due to differences in the antigens, antisera and the techniques used. Due to these discrepancies, major studies are necessary, since a clear definition of antigenic knowledge of this bacterial pathogen is of crucial importance for the development and formulation of appropriate and effective vaccine(s) (Romalde et al. 2005).

Dot blot assays and immunoblot analysis of lipopolysaccharides (LPS) revealed the existence of antigenic diversity in *Tenacibaculum maritimum* and demonstrated that at least 3 major O-serogroups seemingly related to the host species can be detected (Avendaño-Herrera et al. 2004b, 2005b). Thus, the majority of *T. maritimum* isolated from sole in the northwest of Spain and all gilthead sea bream isolates belonged to serotype O1, while all strains isolated from sole in Portugal and southern Spain constituted a serotype (O3), different from those strains isolated from turbot (serotype O2). However, this serological scheme could certainly be extended if further studies, including more strains of *T. maritimum* isolated from different hosts and/or geographical origins, are conducted. The analysis of total and outer membrane proteins revealed that all strains had common bands which are antigenically related.

From a genetic point of view, recent studies employing the random amplification polymorphic DNA polymerase chain reaction (RAPD-PCR) technique revealed the existence of genetic variability within the *Tenacibaculum maritimum* isolated from different marine fish. The strains were separated into 2 main clonal lineages that are strongly associated with the host and/or serogroups described for this pathogen (Avendaño-Herrera et al. 2004d). Interestingly, no

plasmid band was detected in *T. maritimum* isolates using various methods (Avendaño-Herrera 2005). Therefore, the distinct RAPD profiles are not influenced by the presence of extra-chromosomal DNA. This finding constitutes the first evidence of genetic heterogeneity within this fish pathogen, and indicates that the RAPD analysis technique is a useful epizootiological tool for this bacterium.

## DIAGNOSTIC METHODS

The presumptive diagnosis of tenacibaculosis is currently based on the clinical signs of the affected fish, particularly gross external lesions (described above), as well as in the microscopic examination of abundant long, thin, rod-shaped bacteria in wet mounts or Gram preparations obtained from gills or skin lesions of these symptomatic fish. However, the lack of visible *Tenacibaculum maritimum* in early lesions and the relative high incidence of secondary bacterial infections such as those caused by *Vibrio* spp. (Hikida et al. 1979, Kimura & Kusuda 1983, Pépin & Emery 1993, Handler et al. 1997) and saprophytic organisms, mainly ciliated protozoans (McVicar & White 1979, Devesa et al. 1989) such as *Trichodina* and *Uronema* spp. (Chen et al. 1995, Handler et al. 1997), makes visualization difficult and increases the possibility of misdiagnosis.

Because isolation of the bacterium from diseased fish is not always successful, the definitive diagnosis must be supported by the isolation of colonies of *Tenacibaculum maritimum* on appropriate specific media, followed by the determination of at least a limited number of morphological and biochemical characteristics, or by the use of specific molecular DNA-based methods.

The homogeneity of *Tenacibaculum maritimum* facilitates the use of the miniaturized systems API ZYM and API 50CH for its identification (Bernardet & Grimont 1989, Pazos et al. 1993, Bernardet et al. 1994, Chen et al. 1995, Ostland et al. 1999, Avendaño-Herrera et al. 2004b, 2005c). In fact, within the API ZYM gallery, the majority of isolates display a characteristic profile (positive results in the first 11 enzymatic reactions), whereas within the API 50CH not all *T. maritimum* strains were able to produce acid from any carbohydrate substrate. However, all these current microbiological methods based on culture and biochemical characterization are time-consuming. Therefore, procedures for rapid detection and identification of this pathogen are crucial for effective management and disease control in sea farming.

Slide agglutination did not prove to be a useful serological procedure for rapid identification of the bacterium due to the presence of auto-agglutinating



strains (Avendaño-Herrera 2005). Fluorescent antibody techniques have also been used to detect this pathogen with contradictory results (Baxa et al. 1988a, Powell et al. 2004). These serological discrepancies, as well as the antigenic heterogeneity described above, suggest that a PCR methodology should be used as a powerful tool for an accurate identification of the pathogen from plate cultures as well as from tissues.

Two PCR primer pairs have been designed for the detection of *Tenacibaculum maritimum* using the 16S rRNA gene as target. Toyama et al. (1996) selected a pair of primers MAR1 (5'-AATGGCATCGTTTTAAA-3') and MAR2 (5'-CGCTCTCTGTTGCCAGA-3'), positions 190 to 206 and 1262 to 1278, respectively, in the *Escherichia coli* 16S rRNA numbering system, flanking a 1088 bp fragment. This could differentiate *T. maritimum* from the related species *Flavobacterium branchiophilum* and *F. columnare*, as well as from several other pathogenic bacteria. Then, Bader & Shotts (1998) also selected a pair of *T. maritimum* species-specific PCR primers Mar1 (5'-TGTAGCTTGCTACAGATGA-3') and Mar2 (5'-AAATACCTACTCGTAGGTACG-3'), positions 77 to 98 and 1060 to 1081, respectively, from unique sequence stretches within this gene, delimiting a 400 bp DNA fragment. Unfortunately, the authors only tested these primers with pure cultures, and not with infected fish tissues.

Recent research comparing the specificity and sensitivity of these 2 primer pairs demonstrated that the Toyama PCR procedure is the most adequate for an accurate detection of *Tenacibaculum maritimum* in diagnostic pathology, as well as in epidemiological studies of marine tenacibaculosis (Avendaño-Herrera et al. 2004c). Although this method proved useful in detecting acute *T. maritimum* infections in fish, the level of sensitivity was not sufficient to detect the pathogen when present in very low number in asymptomatic or carrier fish. In order to increase its sensitivity, a nested PCR approach was developed and was evaluated in experimentally seeded fish tissues and in field studies (Avendaño-Herrera et al. 2004a,c). The high level of *T. maritimum* found in mucus samples ( $10^3$  CFU ml<sup>-1</sup>) indicates that this non-destructive method is very useful for a specific and rapid (only 7 h) diagnosis of marine tenacibaculosis.

Other molecular methods that couple PCR amplification of 16S rDNA or 16S rRNA genes with a serological procedure or species-specific markers are also available for detecting *Tenacibaculum maritimum*. These include PCR-enzyme-linked immunosorbent assay (PCR-ELISA) (Wilson et al. 2002), reverse transcriptase polymerase chain reaction-enzyme hybridisation assay (RT-PCR-EHA) (Wilson & Carson 2003) and DNA microarray probe (Warsen et al. 2004). These assays have shown sufficient sensitivity for the direct detec-

tion of *T. maritimum* from pure culture, but the effectiveness in field conditions is still unknown. It is important to point out that the RT-PCR assay reported by Wilson & Carson (2003) provided an important advance in the *T. maritimum* detection protocol because it gave a more accurate approximation of live bacterial carriage than conventional PCR.

## PATHOGENESIS OF INFECTION

Despite the significance of *Tenacibaculum maritimum* in the aquaculture industry, relatively little is known about the pathogenicity of this bacterium. Various experimental infection studies have been carried out with varying rates of mortality depending on the different methods used to infect each marine fish species (Wakabayashi et al. 1984, Baxa et al. 1987, Alsina & Blanch 1993, Bernardet et al. 1994, Soltani et al. 1996, Powell et al. 2004, 2005). This makes it difficult to fully understand of the mode of transmission and route of infection of this pathogen. Therefore, most of the information available has been described from naturally infected fish events.

The natural reservoir(s) of the pathogen have not been clarified yet because few data on the ecology of the microorganism exist; however, it can be isolated from sediment, the surface of tanks and from water cultures that have been exposed to infected stocks (Santos et al. 1999).

Some authors reported that natural outbreaks of tenacibaculosis occur a few weeks after transferring fish from hatchery tanks to inshore net cages (McVicar & White 1979, Wakabayashi et al. 1984), suggesting that this agent may gain access to the host by horizontal transmission in seawater. However, recent survival experiments on *Tenacibaculum maritimum* using microcosms under natural conditions demonstrated an inhibitory effect of the natural aquatic microbiota on the growth and survival of this bacterium. In these experiments the pathogen remained culturable for only 5 d (Avendaño-Herrera et al. 2006a). A possible explanation for this is that all cells were lysed by the autochthonous microbiota, followed by rapid degradation of the DNA. However, we cannot rule out some influence of specific bacteriophages and protozoans on the survival of *T. maritimum* in the sea environment. Therefore, these results seem to suggest that seawater would not be an important route of transmission of *T. maritimum*.

It is well known that the primary sites of infection with *Tenacibaculum maritimum* are body surfaces such as the head, mouth, fins, and flanks (see review by Bernardet 1998). This pathogen attaches itself strongly to the external skin and mucus of fish which

do not contain compounds that inhibits the growth of this bacterium (Magariños et al. 1995). The localization of the bacteria within the mucus layer suggests that *T. maritimum* could be part of the autochthonous populations of the fish skin, and therefore the pathogen can remain in the aquatic environment for a long time, utilizing fish mucus as a reservoir (Avendaño-Herrera 2005).

In the case of salmonids, the presence of the bacteria in the eyes and gills, where a necrotizing bronchitis occurs, is not uncommon (Chen et al. 1995, Handlinger et al. 1997). Powell et al. (2004, 2005) inoculated a high concentration of bacteria ( $10^{11}$  to  $10^{12}$  cells per fish) on salmon gills, producing variable mortalities of fish, and they noted that respiratory damage occurred as a consequence of gill abrasion rather than infection with the bacterium. These findings indicate that the gills might not be an especially important route of infection for *Tenacibaculum maritimum*.

*Tenacibaculum maritimum* shows a lack of strict host specificity. Therefore, tenacibaculosis can be a risk for many species of anadromous and marine fish in which the disease has not been yet described. Although both adults and juveniles may be affected by tenacibaculosis, some authors have pointed out differences in the susceptibility of some fish species (i.e. red and black sea bream, sole, sea bass, salmonids and turbot) to the disease on the basis of fish age. Thus, whereas fish with body weights ranging from 2 to 80 g suffer the highest occurrence and a more severe form of the disease, fish above 100 g appear to be resistant (McVicar & White 1979, Wakabayashi et al. 1984, Bernardet et al. 1994, Handlinger et al. 1997, Avendaño-Herrera 2005). This is due to the apparently greater susceptibility of smaller fish to *T. maritimum* (Bernardet et al. 1994), where the severe destruction of the affected tissues could progress from early stages to advanced ulcerative lesions within a few days.

To date, many of the studies on disease transmission support the hypothesis that *Tenacibaculum maritimum* is an opportunistic pathogen that primarily causes extensive skin damage and gill abrasion with subsequent systemic infection.

An increased prevalence and severity of the disease has been reported at higher temperatures (above 15°C) and salinities (30 to 35‰) as well as with low water quality. However, winter outbreaks of tenacibaculosis have also been reported (Wakabayashi et al. 1984, Bernardet et al. 1994, Soltani et al. 1996). In addition to these factors, the disease is influenced by a multiplicity of environmental conditions (i.e. stress, excess of UV light, lack of sand substrate on the tank), management factors (i.e. high density and poor feeding) and host-related factors (skin surface condition)

(McVicar & White 1979, 1982, Wakabayashi et al. 1984, Chen et al. 1995, Magariños et al. 1995, Handlinger et al. 1997). In these adverse conditions, the systemic disease involving different internal organs became more prevalent (Alsina & Blanch 1993, Cepeda & Santos 2002, Avendaño-Herrera et al. 2004b), indicating that *Tenacibaculum maritimum* has strong virulence mechanisms.

With regard to the mode(s) of infection, numerous researchers have investigated different methods to reproduce the disease in various fish species as well as to standardize an effective challenge model. Wakabayashi et al. (1984) and Baxa et al. (1987) demonstrated that bath challenge is not a reliable method of inducing the disease unless the skin is previously scarified or abraded (Bernardet et al. 1994) or the bacterial strain is first passaged twice in fish (Handlinger et al. 1997). However, fatal infections occurred when fish were exposed to topical application of *Tenacibaculum maritimum* culture on the surface of the mouth or tail (Wakabayashi et al. 1984). The intramuscular and intraperitoneal routes have been reported as not being effective in producing tenacibaculosis in experimentally infected red and black sea bream, sea bass or turbot (Wakabayashi et al. 1984, Alsina & Blanch 1993, Pépin & Emery 1993, Avendaño-Herrera et al. 2006b). Using the nested-PCR approach, we demonstrated that most of the *T. maritimum* inoculated in turbot are shed in the water within the first 6 h post-injection (Avendaño-Herrera et al. 2006b). This seems to be the reason why intraperitoneal injection is not effective in reproducing the disease.

Recent research efforts by our group led us to propose of an effective and reproducible infection model in turbot for tenacibaculosis infection. Thus, using prolonged immersion of fish for 18 h with the pathogen at 18 to 20°C, the disease could be easily reproduced with the fish showing the classical signs of tenacibaculosis (Avendaño-Herrera et al. 2006b). This challenge model could be useful for future epizootiological studies and is essential for developing adequate measures to prevent and/or control tenacibaculosis.

In summary, it is conceivable that in the natural environment *Tenacibaculum maritimum* enters the host via a combination of routes including the horizontal transmission via food as described by Chen et al. (1995).

## VIRULENCE MECHANISMS

A first prerequisite for the successful colonization of the host tissue is the ability to adhere. This may lead to a specific or non-specific form of attachment (Ofek & Doyle 1994). Specific adhesion is mediated through

specific compounds on the surface of the bacterium, which bind to receptors present on the host tissue. Non-specific adhesion depends on hydrophobic or ionic interactions between certain structures on the surface of the bacterium and the supporting substrate.

Burchard et al. (1990) reported that the capacity of adhesion of the *Tenacibaculum maritimum* cells significantly increases on substrates with low-critical energy surfaces (hydrophobic). Thus, the bacterium produces a substantial amount of extracellular polymers or 'slime', permitting them to adhere more firmly to hydrophobic surfaces than to those that are hydrophilic. The changes of the cell surface properties do not affect the action of gliding. These characteristics could be an advantage for *T. maritimum* and could explain why the cells systematically adhere onto different parts of the external tissue of fish once an infection has been established.

Adhesion of this pathogen cannot be explained by hydrophobic interactions alone. Sorongon et al. (1991) demonstrated with *in vitro* studies that growth conditions and nutrient availability provoke considerable changes in hydrophobicity and attachment of *Tenacibaculum maritimum*, which were accompanied by changes in the arrays of surface-exposed proteins. In static growth cultures, *T. maritimum* considerably increases the production of diverse polypeptides on the external cellular surface, at higher concentrations than those detected when the bacterium is grown in shaken liquid media.

Hemagglutinating activity, in addition to being a hydrophobic surface property, is sometimes associated with virulence. Pazos (1997) reported that *Tenacibaculum maritimum* cells agglutinate a broad spectrum of erythrocytes. Additional structures such as pili, fimbriae and flagella, known to be involved in adhesion and colonization of other bacteria (Toranzo & Barja 1993, Toranzo et al. 2005), have not been observed in *T. maritimum*. Recent studies have demonstrated that *T. maritimum* isolates, regardless of their serotype, possess capsular material (Fig. 1) (Avendaño-Herrera 2005). Further studies are needed to elucidate its implication in the virulence mechanisms of this fish pathogen.

The adherence of this pathogen to host tissues depends directly on its ability to neutralize or evade the immune system of the fish, such as the bactericidal activity of mucus, and also on its ability to accumulate the nutrients required for its growth. Magariños et al. (1995) reported that *Tenacibaculum maritimum*, regardless of its origin and virulence degree, adhered strongly to the skin mucus of 3 fish species, and resisted its bactericidal properties.

With regard to the production of toxins in *Tenacibaculum maritimum*, Baxa et al. (1988b) conducted *in vivo*

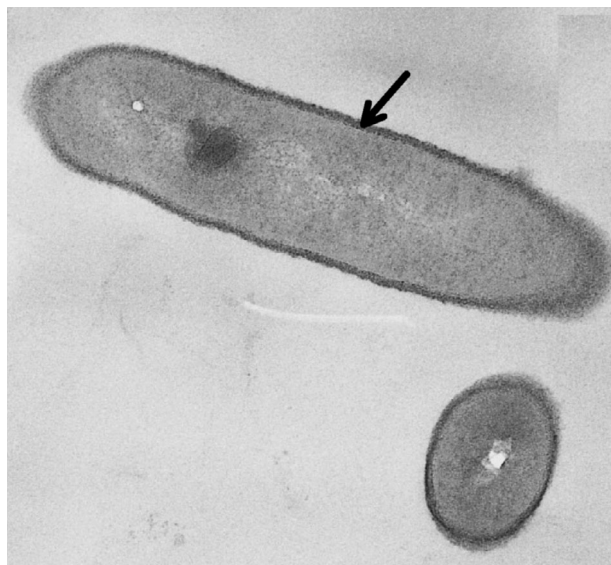


Fig. 1. *Tenacibaculum maritimum*. Transmission electron micrographs of thin sections of cells ( $\times 22000$ ). Arrow indicates a capsular structure strongly stained with ruthenium red

experiments in red and black sea bream, and concluded that the pathogenicity of this pathogen can be attributed to the synergistic interaction of the toxins and enzymes present in the extracellular products (ECP), which could facilitate the alteration and erosion of the host tissue and contribute to colonization and invasion. In fact, the ECP of this species possess very high proteolytic activity with an ability to degrade gelatin, amylase, casein and nucleases (Pazos 1997), as well as a positive cytotoxic activity in various fish cell lines.

It is known that LPS are responsible for some of the characteristic signs of disease in infections due to Gram-negative bacteria. The composition analysis of *Tenacibaculum maritimum* LPS revealed an O-chain composed of a disaccharide that contained an unusual linkage ([R]-2-hydroxyglutaric acid residue), which seems to be unique for this bacterium (Vinogradov et al. 2003). It has been suggested that this O-chain can contribute to the development of biofilms by the bacterium in the fish tissues (Vinogradov et al. 2003).

Another putative virulence factor examined in *Tenacibaculum maritimum* is its capacity to express high-affinity iron-uptake mechanisms, which can compete with the host iron-binding proteins. Avendaño-Herrera et al. (2005c) have demonstrated that this bacterium, regardless of its serotype, grows and multiplies in the presence of the chelating agent ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDHA) using at least 2 different systems of iron acquisition.



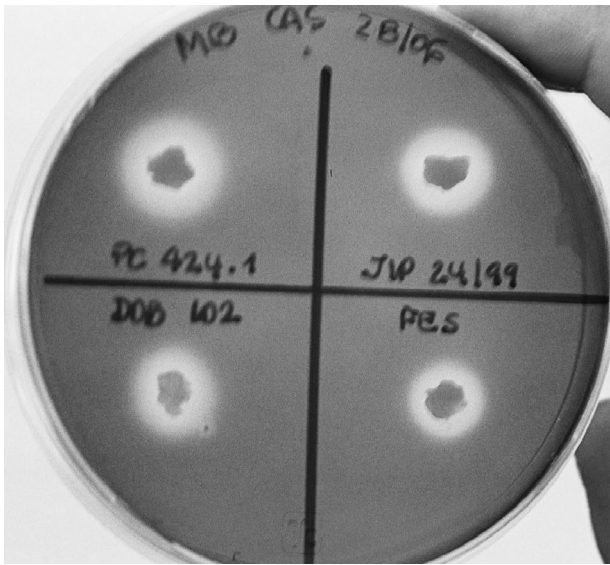


Fig. 2. *Tenacibaculum maritimum*. Modified chromoazuroil S (CAS) agar assay for detecting siderophore production. Siderophore production is evidenced by the halo around the colony

One mechanism involves the production of an unidentified siderophore, which is neither a phenolate nor a hydroxamate type, as demonstrated by the chromoazuroil S (CAS) agar (Fig. 2) and liquid tests. The second mechanism allows the utilization of heme groups (hemin or haemoglobin) as an iron source by direct binding (Fig. 3). In addition, it has also been reported that intraperitoneal injection of hemin before experimental infection increased the lethality of this pathogen (Avendaño-Herrera 2005).

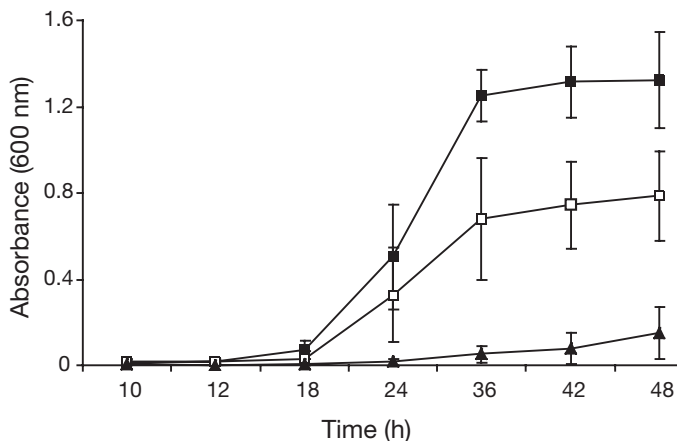


Fig. 3. *Tenacibaculum maritimum*. Average growth of strains utilizing hemoglobin 5 µM (■) and hemin 5 µM (□) as an iron source in FMM medium containing EDDHA. Cells cultured under iron-restricted conditions (▲) were used as negative control. Bars: SD from 3 different strains tested

The lack of plasmids in this pathogen indicate that the expression of the putative virulence factors present in this bacterium must be chromosome-coded.

## TREATMENT AND PREVENTION OF TENACIBACULOSIS

### Chemotherapy

*In vitro* studies on the susceptibility of *Tenacibaculum maritimum* to various chemotherapeutic agents indicate that bacterial strains isolated from different host species and geographical regions exhibit a similar pattern, with respect to susceptibility to nitrofurans, penicillins, erythromycin, tetracyclines, chloramphenicol, trimethoprim, potentiated sulfonamides and fluoroquinolones, and resistance to colistin, kanamycin, neomycin and the quinolones, oxolinic acid and flumequine (Baxa et al. 1988c, Alsina & Blanch 1993, Pazos et al. 1993, Chen et al. 1995, Soltani et al. 1995, Avendaño-Herrera et al. 2004b, 2005a). However, field results were not always similar even if the isolated bacteria were highly sensitive (*in vitro*) to the drug used for treating the condition (Cepeda & Santos 2002).

In practice, variable results were achieved using oral medication and/or bath treatment. The first data on tenacibaculosis control using drugs was reported by McVicar & White (1979) in Scotland. After applying a broad antibiotic spectrum to sole (i.e. furanace, terramycin, kanamycin, Tribissen and Tylan) by oral treatment, the authors did not observe any decrease in mortality. In contrast, the administration of streptomycin sulphate and penicillin by immersion were effective, but both with only short-term results (McVicar & White 1979). It is well known that when infection occurs, fish do not take the medicated feed, and they become anorexic immediately post-infection; thus, bath treatment seems to be more effective than the oral treatment, as reported by Soltani et al. (1995). In addition, these authors showed by means of laboratory and field trials that the administration of amoxicillin and trimethoprim is an effective antimicrobial therapy against *Tenacibaculum maritimum*, producing adequate serum levels when given by oral or immersion procedures to Atlantic salmon and rainbow trout. Treatments with oxytetracycline in salmonids (Handlinger et al. 1997) and furazolidone in turbot (Alsina & Blanch 1993) were also reported to control the disease. Unfortunately, the use of this last drug in fish farms is not permitted in the majority of salmon-farming countries.

Among the drugs used in the past few years in turbot and sole cultures (tetracycline, enrofloxacin, flumequine and potentiated sulfonamides), enrofloxacin proved to be the most useful compound for controlling

*Tenacibaculum maritimum* outbreaks, although the rapid appearance of resistant strains has already been described (Avenidaño-Herrera 2005).

### Other prophylactic treatments

An alternative to drugs would be the use of surface-acting disinfectants administered by immersion. Routine formalin treatments (30 to 40 ppm bath for 6 h) were effective in the control of tenacibaculosis in Dover sole (McVicar & White 1979). However, gill problems associated with the prolonged use of this compound were noted by the same authors. On the other hand, some hatchery managers have expressed concern about user sensitization to formalin and its environmental impact. In addition, this compound is expensive, as well as difficult to use and store. Potassium permanganate has also been used, mainly for water disinfection, to control disease outbreaks in pen-reared sea bass in Malta (Bernardet 1998). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) has recently received attention for its effective control of numerous external pathogens to fish. In the case of *Tenacibaculum maritimum*, Avenidaño-Herrera (2005) recommended the use of H<sub>2</sub>O<sub>2</sub> at a concentration of 240 ppm only as a general disinfection method for treating water culture and the surface of tanks before the introduction of fish.

Manipulation of temperature and/or salinity can be used to reduce the morbidity produced by *Tenacibaculum maritimum* in salmonids; Soltani & Burke (1994) recommended maintaining the temperature around 15°C and salinity below 10 g l<sup>-1</sup>. The appearance of outbreaks can also be avoided or the incidence of the disease can be reduced by controlling fish densities (avoiding overcrowding), reducing stress conditions, and avoiding overfeeding (McVicar & White 1982, Soltani et al. 1996). Sand substrate has been added to tanks to prevent the occurrence of tenacibaculosis and has cured an established disease situation in Dover sole (McVicar & White 1979, 1982). Although all these measures help prevent the disease, modifying husbandry parameters will probably involve related technical problems. Therefore, immunoprophylaxis have become the best way to prevent tenacibaculosis.

### Vaccines

In spite of the severe impact of this disease, relatively few attempts at vaccination have been described and were unknown until the end of the 1990s. To date, there is a general agreement that a vaccine would considerably help to control tenacibaculosis and various groups have established research programs with the

aim of developing a vaccine in Spain and Australia (Håstein et al. 2005). At present, only 1 bacterin is commercially available to prevent turbot mortalities caused by *Tenacibaculum maritimum*. As the disease affects juvenile and adult turbot, the vaccine is applied by bath when the fish are 1 to 2 g, followed by a booster injection when they attain 20 to 30 g. Whereas the relative percentage of survival (RPS) following bath immunization is about 50%, the protection increases above 85% after intraperitoneal (i.p.) booster injection (Toranzo et al. 2004). The regular use of this vaccine in some turbot farms has reduced the incidence of tenacibaculosis. However, the serological diversity described above indicates that the vaccine developed for turbot may not be effective in preventing the tenacibaculosis in other fish species. Therefore, a new tenacibaculosis bacterin specific for cultured sole is currently being developed and evaluated by our research group. We have demonstrated that this confers RPS values higher than 90% in laboratory trials by i.p. injection (Romalde et al. 2005). The studies published on immunization in Australia are scarce. Carson et al. (1993) compared the effectiveness of different bacterins in cultured salmonids and showed that the *T. maritimum* vaccines rendered variable protection levels ranging from 21 to 70%.

Similar to other fish vaccines, it seems that the LPS are the main protective antigen of this pathogen (Salati et al. 2005).

### CONCLUSIONS

We have reviewed the current knowledge about tenacibaculosis in fish and its etiological agent, *Tenacibaculum maritimum*. Despite efforts made in the past 10 years to deal with the problems discussed above, it is obvious that the pathogenesis of *T. maritimum* is a complex, multifactorial process not yet fully understood. There are considerable gaps concerning (1) the route of transmission and reservoir, (2) the survival strategies of this pathogen, an understanding of which would be helpful in developing adequate preventive control programs against marine tenacibaculosis, (3) other possible virulence mechanisms, e.g. the capsule, (4) the pharmacokinetics of drugs in susceptible cultured fish and (5) the establishment of adequate vaccination programmes for economically important marine fish.

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