Departamento de Microbiología y Parasitología
FACULTAD DE BIOLOGÍA
UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

Avances en el conocimiento del patógeno de peces *Tenacibaculum maritimum* : Implicaciones en el diagnóstico y prevención de la enfermedad

Ruben Esteban Avendaño-Herrera

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Memoria que presenta
RUBEN ESTEBAN AVENDAÑO-HERRERA
Para Optar al Grado de
Doctor en Ciencias Biológicas
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Informan: Que la Tesis Doctoral titulada "Avances en el conocimiento del patógeno de peces *Tenacibaculum maritimum* : Implicaciones en el diagnóstico y prevención de la enfermedad" que presenta Don **RUBEN ESTEBAN AVENDAÑO-HERRERA** para optar al grado de Doctor en Ciencias Biológicas, ha sido realizada en el Departamento de Microbiología y Parasitología bajo nuestra dirección, y considerando que se haya concluida autorizamos su presentación para que pueda ser juzgada por el tribunal correspondiente.

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Durante la realización de esta memoria he disfrutado de una **Beca CONICYT-BID del Gobierno de Chile**.

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RESUMEN

RESUMEN

En esta memoria hemos llevado a cabo la caracterización bioquímica, serológica y molecular de *Tenacibaculum maritimum*, agente causal de la tenacibaculosis o flexibacteriosis, enfermedad que causa importantes pérdidas en el cultivo de peces. Asimismo, determinamos la presencia de mecanismos de captación de hierro y los aspectos epizootiológicos de este microorganismo.

Desde el punto de vista fenotípico, *T. maritimum* constituye un grupo homogéneo, pero presenta heterogeneidad antigénica estableciéndose diferentes serotipos, asociados estrechamente con el hospedador. Además, hemos determinado la existencia de variabilidad genética intra-especifica dentro del patógeno, describiendo la existencia de dos líneas clonales mayoritarias, las cuales están relacionados al huésped de aislamiento y/o grupo serológico.

El estudio de los mecanismos de virulencia asociados a la captación de hierro de este microorganismo reveló que *T. maritimum* posee sistemas de elevada afinidad mediados y no mediados por sideróforos, que le permitiría utilizar este componente a partir del hospedador. Se ha demostrado que al menos alguno de éstos sistemas funcionan "*in vivo*".

A nivel de diagnóstico, se ha desarrollado un protocolo no letal sobre la base de la técnica de la PCR anidada, el cual es altamente sensible, específico y de fácil aplicación para la detección de *T. maritimum*, tanto en peces sintomáticos como portadores de la enfermedad.

Los estudios encaminados al posible control y prevención de la enfermedad demostraron que el uso rutinario del quimioterápico enrofloxacina en plantas de cultivo causa una rápida aparición de cepas resistentes a dicho compuesto. Proponemos como método alternativo para prevenir la aparición de los brotes de tenacibaculosis en las piscifactorías la desinfección del agua de cultivo y la superficie de los tanques con peróxido de hidrógeno en una concentración de 240 ppm antes de la introducción de los peces.

El estudio epizootiológico reveló que *T. maritimum* es afectado drásticamente por la microbiota autóctona del agua de mar, por lo que el agua de los tanques de cultivo no sería un importante vector de transmisión de la enfermedad.

ABSTRACT

In this work we conducted the biochemical, antigenical and molecular characterization of *Tenacibaculum maritimum,* the etiological agent of tenacibaculosis or flexibacteriosis. This disease constitutes one of the main problems in marine aquaculture, causing serious mortalities and hence severe economic losses. Likewise the presence of iron uptake mechanisms and the epizootiological aspects of this microorganism were also determined.

From a phenotypic point of view, *T. maritimum* constitutes a homogeneous group, but presents antigenic heterogeneity, revealing the existence of distinct serological groups, strongly correlated with the host species. Moreover, we have demonstrated the existence of genetic variability within *T. maritimum*, being the strains separated into two main groups that are associated with the host species and/or O-serotypes described for this pathogen.

The studies of the virulence mechanisms associated with the ability for iron uptake demonstrated that *T. maritimum* possess high affinity systems to sequester iron from the host, one involving the synthesis of siderophores, and another that allows the utilization of heme groups. Moreover, at least some of these systems works "*in vivo*".

At the diagnostic level, we developed a nested PCR method for the detection of *T. maritimum* using non-destructive samples from different cultured species of marine fish, which is highly sensitive, species-specific and of easy application for the detection of the pathogen. This protocol can be useful to detect the bacterium in symptomatic diseased and asymptomatic carrier fish.

The studies to evaluate the possible control and prevention of the disease demonstrated that the repetitive therapy with the drug enrofloxacin in the fish farms has originated the rapid proliferation of *T. maritimum* resistant strains to this chemotherapeutic agent. Therefore, we propose as an alternative method to prevent the tenacibaculosis outbreak in the fish farm the disinfections of water culture and surface of tanks with hydrogen peroxide at a concentration of 240 ppm before the introduction of fish.

The epizootiological study showed that *T. maritimum* is drastically affect by the natural aquatic microbiota, suggesting that the seawater is not an important route of transmission of tenacibaculosis.

I. INTRODUCCIÓN

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1.1. Generalidades

En los últimos años, la actividad productiva de la cría de especies piscícolas tanto marina como de agua dulce, conocida con el término más general de acuicultura, ha experimentado un crecimiento exponencial muy importante. Así, la acuicultura marina intensiva de peces ha tenido en los últimos años un fuerte desarrollo en todo el mundo, generando un incremento de 733.433 toneladas y retornos cercanos a los 3.142 millones de dólares por toneladas desde 1991 a 2001 (Vannuccini 2003). Sin embargo, al igual que sucede con cualquier otro tipo de cría intensiva animal, la producción de peces desde sus inicios se ha visto limitada por mortalidades masivas de larvas, juveniles y adultos originadas entre otros factores por la aparición de numerosas complicaciones patológicas, entre ellas y de forma particular, problemas de origen bacteriano que suponen en la actualidad un lastre muy importante para el desarrollo presente y futuro de la producción de peces en todo el mundo.

Actualmente están descritas al menos 17 enfermedades de peces asociadas a bacterias que causan importantes pérdidas económicas en el cultivo de peces en el medio marino (Tabla 1) (Toranzo y col. 2005). No obstante, el número de agentes bacterianos asociados específicamente a enfermedades de peces continúa creciendo, incorporándose nuevas especies, como es el caso de *Streptococcus phocae* y *Pasteurella skyensis* o incrementando su incidencia los patógenos existentes, como por ejemplo *Pseudomonas anguilliseptica*, *Streptococcus parauberis* y *Tenacibaculum maritimum*.

Tabla 1. Agentes etiológicos de las principales enfermedades bacterianas que causan importantes perdidas económicas en los cultivos de peces marinos.

Agente	**Enfermedad**	**Hospedadores**
Gram Negativas		
Listonella anguillarum (antes *Vibrio anguillarum*)	Vibriosis	Salmónidos, rodaballo, lubina, anguila, bacalao, ayu y besugo
Vibrio ordalii	Vibriosis	Salmónidos
Vibrio salmonicida	Vibriosis	Salmón del Atlántico y bacalao
Vibrio vulnificus	Vibriosis	Anguilas
Moritella viscosa (antes *Vibrio viscosus*)	Ulcera de invierno	Salmón del Atlántico
Photobacterium damselae subsp. *piscicida* (antes *Pasteurella piscicida*)	Photobacteriosis	Dorada, lubina, seriola y lenguado
Pasteurella skyensis	Pasteurelosis	Salmón del Atlántico
Aeromona salmonicida subsp. *salmonicida*	Forunculosis	Salmónidos y rodaballo
Tenacibaculum maritimum (antes *Flexibacter maritimus*)	Tenacibaculosis	Rodaballo, salmónidos, lenguado, lubina, dorada, pargo, besugo y platija
Pseudomonas anguilliseptica	Pseudomonadiasis	Lubina, anguila, ayu, rodaballo y besugo
Gram Positivas		
Lactococcus garvieae	Estreptococosis o Lactococosis	Seriola y anguila
Streptococcus iniae	Estreptococosis	Platija y lubina
Streptococcus parauberis	Estreptococosis	Rodaballo
Streptococcus phocae	Estreptococcosis	Salmón del Atlántico
Renibacterium salmoninarum	Enfermedad bacteriana del riñón	Salmónidos
Piscirickettsia salmonis	Piscirickettsiosis	Salmónidos
Mycobacterium marinum	Micobacteriosis	Lubina, rodaballo y salmón del Atlántico

La trascendencia económica de la aparición de estas bacterias patógenas en los cultivos, ha incrementado el interés por estudiar estos microorganismos centrándose fundamentalmente en su caracterización taxonómica, bioquímica, fisiológica y molecular, así como en el estudio de los mecanismos de virulencia. El conocimiento de estos aspectos ayuda a desarrollar medidas que permitan rápidamente diagnosticar y contrarrestar la proliferación de estas cepas patógenas en los cultivos, y por lo tanto a la prevención y control de las enfermedades.

1.2. La tenacibaculosis marina

La tenacibaculosis marina es una enfermedad ulcerativa producida por la bacteria filamentosa *Tenacibaculum maritimum* (antes denominado *Flexibacter maritimus*) (Suzuki y col. 2001). Desde sus inicios la enfermedad ha sido descrita bajo diferentes denominaciones, las cuales se encuentran particularmente asociadas a los signos clínicos que se observan en los peces infectados. Entre los principales calificativos se destacan: infección por *Flexibacter*, enfermedad similar a *Flexibacter columnaris* (actualmente *Flavobacterium columnare*), Necrosis de Manchas Negras ("Black Patch Necrosis, BPN"), enfermedad de bacterias deslizantes en peces marinos, Flexibacteriosis Marina, podredumbre de aletas y cola y síndrome de la boca roja (McVicar y White 1979; 1982; Wakabayashi y col. 1984; Alsina y Blanch 1993; Bernardet y col. 1990; Handlinger y col. 1997; Ostland y col. 1999). A pesar de estas repetidas modificaciones en el nombre de la enfermedad, en la actualidad se conoce que en todas estas descripciones la bacteria *T. maritimum* fue el agente causal de las epizootias.

Aunque en un principio se consideró que la enfermedad se restringía a Japón, es a partir de la década de los 90 cuando la tenacibaculosis ha sido detectada en varios países de Europa, siendo considerada de gran importancia económica y constituyendo uno de los principales problemas patológicos limitantes de la producción de peces a nivel mundial.

1.2.1. Patología

Los síntomas clínicos de la infección por *T. maritimum* han sido descritos en detalle por varios autores (McVicar y White 1979; Campbell y Buswell 1982; Wakabayashi y col. 1984; Baxa y col. 1986; Alsina y Blanch 1993; Chen y col. 1995; Handlinger y col. 1997; Ostland y col. 1999; Cepeda y Santos 2002; Avendaño-Herrera y col. 2005: artículo nº 9), tanto en peces cultivados como silvestres y son muy similares a los descritos en peces afectados por la "enfermedad de la columna", producida por *F. columnare* en peces de agua dulce (Campbell y Buswell 1982). Los principales signos de la enfermedad son las erosiones en la superficie de los peces, particularmente hemorragias en la mandíbula (boca roja) y degradación de los tejidos inter-radiales de las aletas caudal y marginal, así como la presencia de úlceras en la piel en distintas zonas del cuerpo (Fig. 1). Internamente, los cambios patológicos descritos son petequias y hemorragia del peritoneo, inflamación y erosión de la cavidad bucal, congestión de meninges, intestino y acumulación de fluído sanguíneo en el abdomen del pez.

Histológicamente, se puede observar acumulaciones de bacterias adheridas a la superficie erosionada, infiltrándose profundamente en tejido conectivo del pez, además de provocar pequeñas zonas de congestión vascular, petequias hemorrágicas y necrosis de tejido. En la boca, se presenta una acumulación bacteriana en los dientes y alrededor de la superficie gingival provocando la invasión del tejido conectivo y ruptura superficial del hueso del maxilar y mandíbula. No obstante, a nivel histológico no se han descrito cambios en órganos internos.

En general las lesiones provocadas por *T. maritimum* dependen de la especie y edad del pez infectado, del lugar geográfico de la infección, de la naturaleza de la infección (natural o experimental) y de la metodología empleada para provocar la enfermedad (inyección intraperitoneal o baño con el patógeno), aunque como se describe a continuación, en la mayoría de los casos es posible observar en los peces síntomas característicos.

En las aletas, las lesiones comienzan con un obscurecimiento de la superficie que se expande rápidamente, y que va dar lugar a la pérdida de superficie epitelial, hemorragia de la dermis y finalmente la destrucción del tejido inter-radial. En la superficie corporal del pez, usualmente las lesiones se inician con la formación de áreas blancas o de color amarillo pálido y/o pequeñas ampollas, debido a la presencia de un gran número de bacteria filamentosas, que van degenerando paulatinamente hasta formar úlceras.

En cuanto a las lesiones en la boca, se presentan como erosiones en la capa dérmica, dejando expuesto el músculo que a su vez, presenta un aspecto hemorrágico. También, en salmón del Atlántico[1] (*Salmo salar*) se han observado placas bacterianas de coloración amarilla asociadas a la superficie de los dientes del premaxilar y mandíbula, al igual que en la superficie de la lengua, vómer y dientes palatinos. Por otro lado, en infecciones experimentales se ha observado lesiones en la cornea de los peces, congestión choroidal y hemorragia sub-choroidal, a veces con ruptura del ojo. Sin embargo, ésta no es una característica general. La existencia de lesiones necróticas en las branquias son menos frecuentes, pero han sido descritas en salmónidos y muestran acumulaciones de bacterias lo cual puede causar problemas de intercambio de oxígeno y equilibrio osmótico.

En general, estos síntomas constituyen los principales signos clínicos de la enfermedad, y es a partir de estas lesiones de donde se ha detectado y aislado *T. maritimum*, aunque también con menos frecuencia, en caso de septicemias severas la bacteria ha sido obtenida a partir de órganos internos de los peces (Alsina y Blanch 1993; Cepeda y Santos 2002; Avendaño-Herrera y col. 2004; 2005: artículos nº 1 y 9).

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[1] El nombre común de cada especie de pez citada en la tesis se halla en: http://www.fishbase.org/search.cfm

Figura 1. Lesiones ulcerativas y hemorrágicas producidas por *T. maritimum* en la superficie externa de lenguado (A), rodaballo (B) dorada (C) y trucha arcoiris (D).

Por otro lado, los peces afectados por tenacibaculosis suelen mostrar natación errática, falta de apetito, adelgazamiento e irregularidades respiratorias. En peces jóvenes la enfermedad progresa rápidamente (2-3 días), originando grandes mortalidades, mientras que en peces adultos la enfermedad se desarrolla de forma más lenta y provoca mortalidades menores, pero con una mayor morbilidad. En general, la muerte de los peces ocurre por la pérdida continua de fluídos corporales y problemas de regulación osmótica, así como por el desarrollo de otras septicemias beneficiadas por el estado de debilidad que alcanzan los peces afectados.

1.2.2. Transmisión y reservorio

El modo de transmisión y ruta de infección de esta enfermedad son todavía desconocidos. Los diferentes ensayos de patogenicidad han demostrado que cuando los peces se exponen a una aplicación tópica de la bacteria (Wakabayashi y col. 1984) o a una combinación de aplicación tópica e inmersión en cultivos bacterianos (Baxa y col. 1987a) se producen mortalidades sensiblemente superiores a las observadas cuando las infecciones experimentales se realizan mediante inyección intraperitoneal. Esto mismo ha sido documentado por Alsina y Blanch (1993), quienes fueron incapaces de reproducir la infección en rodaballo (*Scophthalmus maximus*) cuando administraron el aislado de *T. maritimum* usando inyección intramuscular o dentro del celoma.

Al comparar la efectividad de la aplicación tópica de *T. maritimum*, los resultados descritos son variables y dependen de la zona de aplicación y procedimiento utilizado para la infección. Estudios realizados por Wakabayashi y col. (1984) muestran que la invasión podría ocurrir preferentemente a través de la boca y cola, y no por el tronco del pez, salvo que esta área se encuentre previamente dañada o sin escamas, como ha sido publicado en lubina (*Dicentrarchus labrax*) por Bernardet y col. (1994). Además, se ha establecido que *T. maritimum* posee una gran capacidad de adherencia al mucus de lubina, dorada (*Sparus aurata*) y rodaballo, así como resistencia a la acción bactericida de este material mucoso (Magariños y col. 1995), lo cual podría constituir una puerta de entrada muy importante para el patógeno cuando las condiciones sean óptimas.

Aunque se han detallado casos de tenacibaculosis asociados a problemas branquiales en salmónidos (Chen y col. 1995; Handlinger y col 1997), de acuerdo a los resultados de Powell y col. (2004; 2005), la aplicación tópica del patógeno en las branquias usando concentraciones mayores a 10^{11} células por ml^{-1} provoca mortalidades muy variables y no pueden ser asociadas directamente a la bacteria, sino a la abrasión previa de los arcos branquiales, causando hiperplasia de los filamentos branquiales y reducción de los parámetros respiratorios (tensión arterial de oxígeno y contenido de células transportadoras de oxígeno).

Algunos autores señalan que baños o inmersión de los peces con el patógeno no son métodos seguros para inducir la infección (Wakabayashi y col. 1984; Baxa y col. 1987a), a menos que la superficie externa de los peces sea previamente escarificada (Bernardet y col. 1994). Estudios mas recientes de patogenicidad, sugieren que el agua también podría ser una ruta de infección de la tenacibaculosis, particularmente en salmón del Atlántico, trucha arcoiris (*Oncorhynchus mykiss*) y "greenback flounder" (*Rhombosolea tapirina*) (Soltani y col. 1996; Handlinger y col. 1997). Ambos grupos de investigación, demuestran que la exposición de los peces a dosis de *T. maritimum* entre 10^5 a 10^7 unidades formadoras de colonias (UFC) por ml^{-1} durante un período de 60 a 90 minutos son suficientes para provocar la aparición de los síntomas clínicos en la mayoría de los peces, que luego dan lugar a mortalidades variables (entre 7,2 a 97,5%) dependiendo de la especie y tipo de desafío. Es interesante destacar que las mayores mortalidades fueron obtenidas con cepas de *T. maritimum* que habían sido pasadas varias veces por peces después de su primer aislamiento (Handlinger y col. 1997).

El reservorio natural de *T. maritimum* es aún desconocido, aunque Santos y col. (1999) señalan que la bacteria ha sido aislada de agua, sedimentos y superficie de tanques que habían estado previamente en contacto con peces infectados. Sin embargo, no se puede descartar que la bacteria también utilice como vector y reservorio la superficie externa de los peces (Magariños y col. 1995). De hecho las epizootias ocurridas en Aberdeen (Escocia) por co-habitación de individuos de lenguado común sin historial infectivo con peces traídos de otra área del país (McVicar y White 1982), parece indicar que los peces transferidos eran portadores asintomáticos de la enfermedad. Asimismo, Chen y col. (1995) sugieren que poblaciones de peces silvestres podrían actuar como portadores de *T. maritimum* y servir de vectores de transmisión horizontal de la enfermedad.

Por otra parte, el hecho de que no siempre se detecta infección sistémica y sincronizada en los tanques de cultivo con peces de una misma cohorte, y que la aparición de lesiones externas ocurre sólo en el último estadío de las epizootias hace más difícil determinar el reservorio y vector de infección. Una posibilidad es que *T. maritimum* se encuentre en un estado latente en alguno de estos reservorios como patógeno oportunista, de tal manera que la alteración de cualquier factor ambiental pueda dar lugar a un desencadenamiento de epizootias. Esta última aseveración es apoyada por diversas estudios epidemiológicos (McVicar y White 1979; 1982; Ostland y col. 1999). Hasta ahora no se han realizado estudios que permitan conocer el reservorio primario en la ruta de infección y tampoco el papel que juega el agua de cultivo en la epidemiología del patógeno (Capítulo VI: artículo nº 6).

1.2.3. Rangos de hospedadores y distribución geográfica

El aislamiento de *T. maritimum* como agente etiológico responsable de la enfermedad se describió por primera vez en alevines de pargo (*Pagrus major*) y besugo *(Acanthopagrus schlegeli)* (Masumura y Wakabayashi 1977; Hikida y col. 1979) en Japón, donde años más tarde se describieron también brotes de esta enfermedad en otras especies como solla japonesa (*Paralichthys olivaceous*), seriola *(Seriola quinqueradiata*), chivo (*Aluterus monoceros*) y torafugu (*Takifugu rubripes*) (Baxa y col. 1986; Baxa 1988). Posteriormente, el patógeno se ha aislado a partir de numerosas especies de peces cultivados y salvajes, mostrando la carencia de un hospedador específico (Bernardet y col. 1994), y en regiones geográficas muy diversas (Fig. 2).

En Europa, la enfermedad fue descrita por primera vez en Escocia, donde causó importantes mortalidades en cultivos de lenguado común, siendo denominada Necrosis de Manchas Negras (McVicar y White 1979; 1982). A pesar de la severidad de la enfermedad, el agente causal fue aislado por Campbell y Buswell (1982) y descrito como "*Flexibacter columnare*-like" después de cuatro años desde los primeros casos de problemas patológicos. Con posterioridad, la bacteria fue identificada como *Flexibacter maritimus* (Bernardet y col. 1990). A partir de la década de los noventa la tenacibaculosis constituye un problema preocupante para el cultivo de una gran diversidad de especies piscícolas de agua de mar, como el rodaballo, salmón del Atlántico, salmón Coho (*Oncorhynchus kisutch*), lenguados (*Solea solea* y *Solea senegalensis*), dorada y lubina en distintas áreas geográficas (Devesa y col. 1989; Bernardet y col. 1990; 1994; Alsina y Blanch 1993; Pazos y col. 1993; Santos y col. 1999; Bader y Starliper 2002, Cepeda y Santos 2002).

La tenacibaculosis también ha sido descrita en el continente Americano, específicamente en la costa Sur de California (Estados Unidos), donde ha afectado al salmón Chino (*Oncorhynchus tschawytscha*) cultivado en jaulas marinas, al igual que a poblaciones capturadas de corvina blanca (*Atractoscion nobilis*), anchova del Norte (*Engraulis mordax*) y sardina del Pacífico (*Sardinops saxax*) (Chen y col. 1995). También Ostland y col. (1999) han descrito algunos casos de la enfermedad en salmón del Atlántico cultivado en el Noroeste de Canadá (British Columbia).

En el hemisferio sur, *T. maritimum* ha sido identificado como patógeno en diversos cultivos de peces de Tasmania (Australia), incluyendo salmón del Atlántico, trucha arcoiris, "greenback flounder", trompeta rayada (*Latris lineata*), "yellow eye-mullet" (*Aldrichetta forsteri*) y besugo negro (*Acanthopagrus butcheri*) (Soltani y Burke 1994; Handlinger y col. 1997). Otra área geográfica es Chile, donde se sospecha que la bacteria causante de la tenacibaculosis puede ser responsable de epizootias ocurridas en piscifactorías de salmón del Atlántico y rodaballo. Sin embargo, hasta la fecha no han sido obtenidos aislados de *T. maritimum*.

Figura 2. Distribución geográfica de *Tenacibaculum maritimum*. Áreas donde han sido descritos brotes infecciosos y con el aislamiento del patógeno(●) y otras zonas con masivas mortalidades de peces, los cuales presentan síntomas presuntamente de tenacibaculosis, pero sin aislamiento del agente etiológico de la enfermedad (●).

1.2.4. Diagnóstico de la enfermedad

Los signos clínicos señalados en el apartado de patología (ver 1.2.1) y los cambios observados en la piel no constituyen un diagnóstico específico de la tenacibaculosis, ya que pueden ser producidos por otras causas muy diversas (McVicar y White 1979). Además, las lesiones ulcerosas en la epidermis favorecen la entrada de otras bacterias patógenas como *Vibrio* spp. (Kimura y Kusuda 1983), particularmente de la especie *V. splendidus* (Handlinger y col. 1997) y organismos saprofíticos, principalmente protozoos ciliados (McVicar y White 1979; Devesa y col. 1989) como *Trichodina* y *Uronema* (Chen y col 1995; Handlinger y col. 1997), razón por la cual *T. maritimum* aparece a menudo en infecciones mixtas.

Además, como se señaló previamente, la dificultad de observar las bacterias al inicio de las infecciones causadas por *T. maritimum*, junto con la alta incidencia de patógenos secundarios genera en la mayoría de los casos un diagnóstico erróneo o tardío de la enfermedad. Por consiguiente, los síntomas externos de los peces en combinación con el examen microscópico de preparaciones frescas a partir de las lesiones pueden usarse sólo para el diagnóstico presuntivo de la enfermedad. Por ello, salvo la utilización de algunos métodos moleculares, sólo el aislamiento de *T. maritimum* en medios específicos y la obtención de cultivo puro, seguido de la identificación mediante distintos métodos pueden confirmar el diagnóstico.

El aislamiento primario de *T. maritimum* se realiza a partir de la superficie externa del pez en medios sólidos, no selectivos, que contienen bajas concentraciones de nutrientes y preparados con agua de mar. En caso de suponer bajas densidades del patógeno, la muestra se enriquece en medio líquido y mediante pases sucesivos se purifica en medios de cultivos sólidos. El período de incubación en la mayoría de los casos fluctúa de 2 a 4 días a temperaturas que oscilan de 20 – 25ºC.

Históricamente, diversos medios de cultivo han sido empleados para obtener un buen crecimiento de *T. maritimum*, y aunque el medio más utilizado para el aislamiento del microorganismo ha sido el agar descrito por Anacker y Ordal (1959) preparado con agua de mar (Hikida y col. 1979; Bullock y col. 1986; Wakabayashi y col. 1984; 1986; Handlinger y col. 1997), el patógeno también ha sido recuperado en Agar Marino (Campbell y Buswell, 1982; Alsina y Blanch 1993) y medio de Hsu-Shotts suplementado con sulfato de neomicina y polimicina (Chen y col. 1995). Otros medios, como el Triptona-Casaminoácidos-Levadura (Wakabayashi y col. 1984; 1986; Ostland y col. 1999), Triptona-Extracto de Levadura-Sales (Toyama y col. 1996), Triptona-Levadura (Bader y Shotts 1998) y 1/5 LBM [triptona y extracto de levadura disueltas en agua de mar artificial] (Suzuki y col. 2001) han sido descritos para el cultivo en laboratorio y el estudio de algunas características fisiológicas de *T. maritimum*, aunque no son utilizados de rutina para el aislamiento de la bacteria a partir de muestras de campo.

Pese a que todos los medios señalados anteriormente son apropiados para el cultivo rutinario de *T. maritmum*, el medio *Flexibacter maritimus* (FMM) ha sido descrito como el más efectivo para el aislamiento y recuperación del patógeno a partir de peces enfermos (Pazos y col. 1996). Esto se debe a que este medio no facilita el crecimiento de diferentes bacterias heterótrofas que invaden las lesiones ulcerosas, haciendo más fácil distinguir las colonias de *T. maritimum* de las otras especies bacterianas. Además, en este medio sólido las colonias de *T. maritimum* son planas, delgadas, con bordes irregulares, color amarillo y se adhieren ligeramente al agar (Fig. 3), pero igualmente se debe realizar la confirmación del microorganismo mediante alguno de los métodos que se describen a continuación.

Figura 3. Colonias típicas de aislados de *T. maritimum* crecidos en el medio *Flexibacter maritimus* (FMM)

1.2.4.1. Métodos convencionales

Se basan en el estudio de una serie de características clásicas de la bacteria (morfológica, fisiológica y bioquímica), entre ellas, su forma filamentosa y carácter Gram negativa, movimiento deslizante característico, crecimiento a diferentes concentraciones de agua de mar, absorción de rojo Congo, indicativo de la producción de glucano galactosamina y ausencia de pigmento del tipo flexirrubina (ver Tabla 3). Estas pruebas, en combinación con la utilización de sistemas convencionales como el API ZYM y API 50 CH son de gran utilidad para la identificación de *T. maritimum* (Pazos y col. 1993; Chen y col. 1995; Bernardet y Grimont 1989; Bernardet y col. 1990; Ostland y col. 1999; Avendaño-Herrera y col. 2004: artículo nº 1). Sin embargo, al contrario de lo que ocurre con otros patógenos marinos (Toranzo y col. 2005), la utilización única de estos sistemas multipruebas no son suficiente para la identificación de *T. maritimum*.

En el caso de las galerías API 50 CH, si bien el patógeno no es capaz de hidrolizar ninguno de los carbohidratos probados, siendo un patrón característico para cepas de *T. maritimum*, este resultado es coincidente al mostrado por otros patógenos de peces fenotípicamente similares a *T. maritimum* y que pertenecen a los grupos *Cytophaga-Flavobacterium-Bacteroides* (Bernardet y Grimont 1989), lo cual puede llevar a graves errores de tipificación. En cuanto, a los patrones de actividad enzimática observadas al analizar aislados de *T. maritimum* mediante el sistema API ZYM, los resultados no sólo muestran pequeñas diferencias cualitativas relacionadas con el origen de las cepas, sino que la bibliografía evidencia notables incongruencia en los perfiles de degradación de las enzimas lipasa y α-quimotripsina, incluso usando las mismas cepas de referencias (Pazos y col. 1993; Chen y col. 1993; 1995; Bernardet y Grimont 1989; Bernardet y col. 1990; Ostland y col. 1999). Además, Ostland y col. (1999) describen aislados de *T. maritimum* con actividad α- y β-glucosidada, característica poco común para el patógeno (Bernardet y Grimont 1989).

Sin embargo, aunque estos métodos tradicionales en *T. maritimum* han sido ampliamente utilizados debido a que son de bajo costo, sencillos de emplear y ofrecen resultados fiables, el tiempo necesario para llevar a cabo la identificación del patógeno supera los tres-cuatro días, demorando su diagnóstico y por lo tanto el control de la enfermedad.

1.2.4.2. Métodos serológicos

En general, las técnicas serológicas utilizadas para la detección y diagnóstico del patógeno, emplean anticuerpos policlonales obtenidos en conejos frente a células formolizadas de *T. maritimum*. Los primeros en aplicar métodos de este tipo fueron Wakabayashi y col. (1984), quienes usando la técnica de aglutinación en portaobjetos observaron reacciones cruzadas entre diferentes cepas de *T. maritimum* aisladas de besugo y pargo en Japón. No obstante, este procedimiento basado en la reacción antígeno-anticuerpo, y que es ampliamente utilizado para la identificación de diversos microorganismos patógenos de peces (Toranzo y col. 2005), tiene un uso limitado en el caso de *T. maritimum* debido a la existencia de cepas autoaglutinantes.

Si bien, técnicas de inmunodifusión en geles y dot-blot han permitido la eficiente identificación del microorganismo (Ostland y col. 1999; Pazos 1997), presentan el inconveniente de ser un procedimiento tedioso, lento y requiere tener la bacteria aislada en medios de cultivo. Además, cabe denotar que Ostland y col. (1999) tan sólo usaron el antisuero obtenido frente a la cepa tipo de referencia, quedando por determinar si los resultados son reproducibles con más cepas de *T. maritimum* aisladas a partir de nuevas especies y/o orígenes geográficos o empleando antisueros obtenidos de otros aislados como ha sido demostrado en nuestros propios estudios (Capítulo III; artículos nº 1 y 2).

Otros métodos como, los ensayos de inmunofluorescencia utilizando anticuerpos anti-*T. maritimum* marcados con fluoresceína, permiten un diagnóstico rápido de la enfermedad (Baxa y col. 1988a). Estos autores demostraron la utilidad de esta prueba como procedimiento de diagnóstico en peces que externa o internamente presentaban los síntomas clínicos de la tenacibaculosis, así como una mayor sensibilidad comparado con los métodos convencionales de cultivo en medios sólidos. Sin embargo, Powell y col. (2004) usando la misma técnica de inmunofluorescencia, pero en forma indirecta (IFAT) observaron resultados variables dependiendo de la cepa de *T. maritimum* empleada para causar la infección experimental, lo cual parece indicar que existen diferencias en la recubierta superficial o glicocálix de la bacteria.

Por lo tanto, la immunofluorescencia no es una técnica utilizada de forma rutinaria en la identificación de *T. maritimum*, probablemente debido a los requerimientos necesarios (antisueros marcados, microscopio de fluorescencia, personal especializado, etc.), la ambigüedad en la especificidad de la técnica y a que las muestras de tejidos de los hospedadores infectados deben ser trasportados en forma inmediata desde la piscifactoría al laboratorio.

Por otro lado, los ensayos de detección de *T. maritimum* mediante técnicas de inmunohistoquímica aplicadas a tejidos infectados en muestras de campo, no han demostrado la misma eficiencia que los ensayos de inmunofluorescencia descritos por Baxa y col. (1988a). De hecho, Ostland y col. (1999) al muestrear salmónidos cultivados con síntomas clínicos y aparentemente sanos observó reacción cruzada de los anticuerpos anti-*T. maritimum* con otros microorganismos oportunistas presentes en las muestras.

Finalmente, en el caso de *T. maritimum* estos métodos de diagnóstico necesitan una mayor evaluación, tanto de sensibilidad como de especificidad, más aún considerando la falta de concordancia en los grupos serológicos establecidos dentro de la especie (Wakabayashi y col. 1984; Pazos 1997; Ostland y col. 1999).

1.2.4.3. Métodos moleculares

El desarrollo de técnicas moleculares como la Reacción en Cadena de la Polimerasa (PCR) y la combinación de esta tecnología con métodos serológicos o marcajes especie-específico han ayudado a solucionar la mayoría de los problemas descritos anteriormente (ver 1.2.4.1 y 1.2.4.2). Los primeros estudios genéticos en *T. maritimum* fueron realizados por Woese y col. (1990), quienes obtuvieron la secuencia completa de la región del ácido dexosirribonucleico (ADN) que codifica para el 16S rARN en *T. maritimum*, y que podría ser utilizado para la clarificación de la posición filogenética del patógeno, así como para constituir la base para el diseño de diferentes métodos basados en el ADN.

Basándose en estas secuencias disponibles en la base de datos GenBank (www.ncbi.nlm.nih.gov acceso número M64629), Toyama y col. (1996) desarrollaron una pareja de cebadores que amplifica un fragmento de un tamaño de 1088 pares de base (pb), el cual es específico y permite diferenciar *T. maritimum* de especies bacterianas fenotípicamente similares (*F. columnare* y *Flavobacterium branchiophilum*), así como otras bacterias patógenas de peces. Posteriormente, otros autores han desarrollado una nueva pareja de iniciadores, seleccionando una secuencia más estrecha dentro del 16S rARN de la bacteria, y que amplifica un fragmento cromosómico específico de 400 pb (Bader y Shotts 1998).

A pesar del potencial de ambos protocolos de PCR, Toyama y col. (1996) y Bader y Shotts (1998) únicamente estudiaron la detección de *T. maritimum* a partir de cultivos bacterianos crecidos en medio sólidos y no aportan ninguna información sobre la sensibilidad de cada pareja de cebadores. Además, se desconoce la especificidad y eficiencia en el diagnóstico usando muestras ambientales y/o tejidos de peces.

Por ello, en esta memoria hemos comparado la especificidad y sensibilidad de los protocolos de PCR descritos para la identificación de *T. maritimum*, tanto en ensayos "*in vitro*" como en estudios de campo y en base al protocolo de Toyama y col. (1996) se ha desarrollado un procedimiento de PCR-anidada ("Nested-PCR") no letal, altamente sensible, específico y de fácil aplicación que permita la rápida detección de *T. maritimum* tanto en peces sintomáticos como portadores de la enfermedad (Capítulo V: artículo nº 4). Cabe señalar, que este procedimiento consiste en la amplificación previa del ADN usando una pareja de cebadores universales, los que reconocen la región conservada del 16S rARN y el producto de amplificación obtenido es empleado en una segunda ronda de PCR usando la secuencia de oligonucleotidos específica para la detección de especie en estudio.

Por otra parte, el grupo de investigación dirigido por el Dr. Carson en la Universidad de Tasmania (Australia) ha diseñado dos ensayos de alta sensibilidad para la detección de *T. maritimum*, los cuales combinan herramientas moleculares como la técnica de Transcripción Reversa-Reacción en Cadena de la Polimerasa (RT-PCR) con ensayos de hibridización enzimática usando un proceso denominado Detección de Producto Amplificado Inmovilizado en un Sistema de una Fase (DIAPOPS, Nalge Nunc International) (Wilson y col. 2002; Wilson y Carson 2003). A pesar de que ambos ensayos permiten la identificación del patógeno previamente aislado en cultivo puro, no han validado su funcionamiento con muestras de campo. Interesantemente, el método descrito por Wilson y Carson (2003) podría tener un gran potencial para la monitorización de células vivas de *T. maritimum* en poblaciones de peces y no bacterias muertas o fragmentos de ADN del patógeno como ocurre con los métodos convencionales y modificados de PCR.

Recientemente, se ha desarrollado un sistema de diagnóstico basado en la construcción de una micromatriz que contiene sondas de oligonucleotidos específicas para *T. maritimum* y que permiten la detección del ADN amplificado previamente mediante la técnica de PCR (Warsen y col. 2004). Este ensayo se apoya en lo que se conoce como "Paradoja de los Polimorfismos", y que consiste en seleccionar una región del 16S rADN que posea un alto grado de variación evolutiva para la construcción de una sonda específica, por lo que la secuencia del gen podría inclusive ser propensa a frecuentes mutaciones. Según estos autores, el ensayo permite discriminar hasta 15 diferentes patógenos bacterianos de peces, pero a nuestro parecer el empleo de este sistema para diagnóstico no es totalmente claro, ya que sólo se empleó una cepa de *T. maritimum* y no repara en la posibilidad de que dentro de esta especie bacteriana exista diversidad intraespecífica como muestran los resultados incluídos en esta memoria (Capítulo IV: artículo nº 3). Por otro lado, Warsen y col. (2004) evaluaron el potencial de su técnica sólo en cultivos puros de *T. maritimum*.

Como queda de manifiesto, la mayoría de las técnicas moleculares publicadas hasta ahora en *T. maritimum* tienen algún tipo de inconveniente, a pesar de esto, la técnica de la

PCR ha sido considerada una herramienta fundamental para la identificación específica de la totalidad de agentes patógenos conocidos y responsables de graves pérdidas en el cultivo de peces, debido a que permiten un diagnóstico rápido y por lo tanto el control de la enfermedad (ver revisiones de Cunningham 2002; Osorio y Toranzo 2002)

1.2.5. Factores que favorecen la aparición de la enfermedad

Algunos autores han relacionado la prevalencia y severidad de la tenacibaculosis con las fluctuaciones en las condiciones ambientales del agua. Así, el incremento de la temperatura del agua (>15ºC), aumento de la salinidad y una baja calidad del agua parecen favorecer la infección por *T. maritimum* (Campbell y Buswell 1982; McVicar y White 1982; Wakabayashi y col. 1986; Handlinger y col. 1997). Sin embargo, no se puede descartar la existencia de infecciones severas por *T. maritimum* en invierno, cuando las temperaturas del agua son mas bajas. De hecho, Wakabayashi y col. (1984) y Bernardet y col. (1994) lo demuestran, aislando este microorganismo como agente responsable de las infecciones ocurridas en los meses de invierno en los cultivos de alevines de pargo y besugo en Japón y en piscifactorías de lubina en Francia. En ambos trabajos no se descarta el estrés como causa principal de las epizootias, ya que la aparición de la enfermedad coincide con unas dos a tres semanas después del movimiento de los peces al mar. Estudios realizados por Soltani y Burke (1994) sugieren que dentro de la especie *T. maritimum* existe un crecimiento condicionado por la temperatura del agua de mar, asociando este factor a la procedencia geográfica de la bacteria. De esta forma, los investigadores denotan que los aislados procedentes de Australia crecen a temperaturas aún mas bajas (8ºC) que aquellas cepas de *T. maritimum* de origen japonés y europeas (15ºC).

Aunque los factores ambientales son de crucial importancia en el potencial patogénico de *T. maritimum*, la manifestación de la tenacibaculosis está profundamente influenciada por otros factores. Si bien, la enfermedad no discrimina entre peces jóvenes y adultos, son en la mayoría de los casos los peces jóvenes (0,5 a 100 g) los que sufren una forma más severa de la enfermedad (McVicar y White 1979; Wakabayashi y col. 1984; Bernardet y col. 1994; Cepeda y Santos 2002). Esto está apoyado por Soltani y col. (1996) y Handlinger y col. (1997), quienes al realizar infecciones experimentales en trucha arcoiris entre 50 a 140 g, observaron que las mayores mortalidades ocurren en los grupos de peces más pequeños.

Como se señaló anteriormente, el estrés originado durante el proceso de transferencia de los peces desde los criaderos a jaulas (Wakabayashi y col. 1984; Pépin y Emery 1993), junto a los traumas físicos y abrasiones mecánicas originadas por la manipulación inadecuada predispone a los peces a la infección por *T. maritimum* (Chen y col. 1995). En salmónidos el exceso de radiación ultravioleta y la existencia de patologías en las branquias, también han sido considerados agentes desencadenante de la enfermedad (Handlinger y col. 1997). McVicar y White (1982), denotan que la ausencia de substrato arenoso en los tanques de lenguado beneficia el establecimiento de la tenacibaculosis. Otros factores señalados son la superpoblación de peces, que causan lesiones por fricción y en algunos casos canibalismo y acentúan los estados de estrés de los peces, debilitando así el sistema inmunitario y favoreciendo la actuación de parásitos externos (Chen y col. 1995).

1.3. *Tenacibaculum maritimum*

1.3.1. Posición taxonómica

En 1977, Masumura y Wakabayashi describieron por primera vez esta bacteria deslizante como la causante principal de mortalidades severas de peces juveniles en Japón. Posteriormente, las cepas bacterianas aisladas a partir de lesiones externas y riñón fueron caracterizada por Hikida y col. (1979) y de modo más amplio por Wakabayashi y col. (1984), quienes sugirieron el nombre de *Flexibacter marinus*. Sin embargo, como el epíteto *marinus* había sido previamente utilizado en la especie *Vibrio marinus* se le asignó a la especie el nombre de *Flexibacter maritimus* (Wakabayashi y col. 1986), y una cepa aislada de pargo japonés se depositó en la Colección Nacional de Bacterias Industriales y Marinas (NCIMB, Escocia) como cepa tipo (NCIMB 2154) junto con una cepa adicional aislada de besugo japonés (NCIMB 2153).

A partir de ese momento, la taxonomía de la especie ha sufrido repetidas modificaciones creándose en ocasiones nuevas especies. Por ejemplo, el nombre *Cytophaga marina* fue propuesto para el mismo organismo en el Bergey's Manual of Systematic Bacteriology con el aislado de besugo japonés (NCIMB 2153) como cepa tipo (Reichenbach 1989). Bernardet y Grimont (1989) mediante estudios de hibridización ADN-ADN demostraron que ambas cepas pertenecían a la misma especie y se le dio prioridad a la nomenclatura de *Flexibacter maritimus* siendo reconocida la cepa NCIMB 2154 como cepa tipo (Holmes 1992). Además, este autor sugiere que el organismo debería permanecer como *Flexibacter maritimus* hasta que estudios genéticos más completos permitieran diferenciar la rama filogenética *Cytophaga-Flexibacter-Flavobacterium*.

En el año 2001, Suzuki y col. basándose en análisis de la secuencia del gen *gyrB* y la secuencia de aminoácidos de la proteína GyrB de aislados pertenecientes a los grupos *Cytophaga-Flavobacterium-Bacteroides*, han propuesto la inclusión de *Flexibacter maritimus* en un nuevo género denominado *Tenacibaculum* (Te.na.ci.ba'cu.lum. L. adj. n. *tenax* adhesión rápida; L. neut. n. *baculum* bastón), que incluiría además al patógeno de peces *Flexibacter ovolyticus* (Hansen y col. 1992), denominándose las dos especies como *Tenacibaculum maritimum* y *Tenacibaculum ovolyticum* respectivamente. Además, estos autores describen dos nuevas especies dentro de éste género, *Tenacibaculum mesophylum* y *Tenacibaculum amylolyticum*, las cuales incluyen cepas aisladas de esponja y macroalgas en Japón.

En su raíz latina el género es definido como "microorganismos en forma de bacilo, los cuales se adhieren a la superficie de organismos marinos" y la especie tipo seleccionada por Suzuki y col. (2001) fue *T. maritimum*. Recientemente, otras dos especies bacterianas han sido incluídas dentro del género, recibiendo los nombres de *Tenacibaculum skagerrakense* (Frette y col. 2004) y *Tenacibaculum lutimaris* (Yoon y col. 2005). Cabe señalar que una propiedad característica de este nuevo género es que las cinco especies marinas contienen menaquinona-6 (MK-6) como sistema respiratorio quinona y la relación de bases nitrogenadas guanina-citosina varia de 30,3 – 35,2 mol% (G+C) (Suzuki y col. 2001; Frette y col. 2004; Yoon y col. 2005). En la Tabla 2 se muestran las principales características de las distintas especies del género *Tenacibaculum*.

Tabla 2. Características comunes y diferenciales descritas en las especies incluidas dentro del género *Tenacibaculum* (Suzuki y col. 2001; Frette y col. 2004; Yoon y col. 2005). + , prueba positiva; – , prueba negativa; (d), resultado débil; v, resultado variable; ND, no determinado; +*, aparecen en cultivos antiguos (Wakabayashi y col. 1986).

Características	***T. maritimum***	***T. amylolyticum***	***T. mesophilum***	***T. ovolyticum***	***T. skagerrakense***	***T. lutimaris***
Origen	Peces marinos (Japón)	Macroalga (Japón)	Esponja y Macroalga (Japón)	Huevos de Halibut (Noruega)	Pelágica (Dinamarca)	Marea baja (Korea)
Tamaño celular (µm)	0,5 x 2 – 30	0,4 x 2-4	0,5 x 1,5 – 10	0,5 x 2 – 20	0,5 x 2-15	0,5 x 2-10
Presencia de células esféricas	+*	Ocasional	Ocasional	ND	Frecuentes	Ocasional
Morfología de la colonia						
Forma	Plana y delgada	Circular	Irregular	Irregular	Irregular	Irregular
Diámetro a los 5 d (mm)	< 5	23 – 27	30 – 60	ND	5 – 20	10 – 20
Color	Amarillo pálido	Amarilla	Amarilla	Amarillo pálido	Amarillo vivo	Amarillo pálido
Rango de salinidad (%)						
NaCl	No crece	(3 d)	1 – 7	No crece	No crece	2 – 3
Agua de mar	30 – 100	50 – 100	10 – 100	70 – 100	25 – 150	ND
Rango de pH	5,9 – 8,6	5,3 – 8,3	5,3 – 9,0	5,9 – 8,6	6,0 – 9,0	7,0 – 8,0
Temperatura (ºC)						
Crecimiento	15 – 34	20 – 35	15 – 40	4 – 25	10 – 40	10 – 39
Óptima	30	27 – 30	28 – 35	ND	25 – 37	30 – 37
Crecimiento en:						
L-Leucina	–	–	–	–	d	–
Sucrosa	–	–	–	–	+	–
DL-Aspartato	–	–	+	–	+	–
Degradación de:						
Quitina	–	–	–	+	–	ND
Almidón	–	+	–	–	+	–
Tween 80	+	+	+	+	–	–
Reducción de nitrato	+	D	–	+	+	v (–)
Contenido G + C (mol %)	31,3 – 32,5	30,9	31,6 – 32	30,3 – 32	35,2	32,3 – 32,8

1.3.2. Caracterización morfológica y bioquímica

Las características fenotípicas y bioquímicas útiles para la identificación de *T. maritimum* han sido descritas previamente por diversos autores (Wakabayashi y col. 1984; 1986; Baxa y col. 1986; 1987b; Bernardet y Grimont 1989; Bernardet y col. 1990; 1994; Chen y col. 1995; Ostland y col. 1999; Suzuki y col. 2001). En la mayoría de los estudios muestran la existencia de una alta homogeneidad entre los aislados incluídos dentro de la especie *T. maritimum*. Sin embargo, resultados variables han sido descritos en propiedades como la reducción de nitratos, producción de H_2S (Wakabayashi y col. 1986; Chen y col. 1995; Avendaño-Herrera y col. 2004: artículo nº 1), degradación de gelatina (Suzuki y col. 2001), crecimiento en N-acetilglucosamina, sacarosa y D-ribosa (Gosink y col. 1998) y crecimientos en medios de cultivos preparados con diferentes concentraciones de agua de mar (Baxa y col. 1986). Probablemente, las diferencias sean debido a las condiciones de crecimiento, particularmente a la disponibilidad de diversas fuentes de carbono que dependen del medio basal empleado en la caracterización bioquímicas (Suzuki y col. 2001). La Tabla 3 muestra las principales características fenotípicas y bioquímicas del patógeno de peces marino *T. maritimum*.

Es importante destacar que la descripción del microorganismo es idéntica a la dada para *F. maritimus* por Wakabayashi y col. (1986). De esta forma, *T. maritimum* es un bacilo alargado (2-30 µm x 0,5 µm), Gram-negativo, aerobio estricto y con movimiento deslizante. La bacteria generalmente es filamentosa, aunque después de pocos días de su cultivo las células se acortan y tienden a hacerse esféricas, apareciendo una forma degenerativa denominada esferoplasto (aproximadamente 0,5 µm de diámetro). Estas formas esféricas no son capaces de multiplicarse en un nuevo medio de cultivo líquido. El microorganismo no forma microcistos, esporas ni vesículas de gas y no se han observado la formación de cuerpos fructiferantes. Además, *T. maritimum* es oxidasa y catalasa positiva y absorbe el colorante rojo Congo, que indica la producción del glucano galactosamina. También, produce pigmentos de tipo carotenoide, principalmente zeaxantina y en menor cantidad *β*-criptoxantina y *β*-caroteno, pero carece de pigmentos de tipo flexirrubina.

La temperatura de crecimiento del microorganismo varía entre los 15 y 34ºC y rangos de pH entre 5,9 y 8,6. La bacteria es un microorganismo estrictamente marino y los medios de cultivos requieren al menos 30% de agua de mar para permitir el crecimiento de *T. maritimum*. Por lo tanto, la adición de cloruro de sodio a medios de cultivos no es suficiente para soportar el crecimiento del patógeno, ya que la bacteria necesita de sales inorgánicas que contengan los iones Ca^{+2} y Mg^{+2} para favorecer su crecimiento, mientras que el SO_4^{+2} es ligeramente inhibitorio.

1.3.3. Caracterización serológica

A nivel antigénico, *T. maritimum* ha sido descrito inicialmente como una especie homogénea, debido a que todos los aislados, independiente de su origen, comparten un antígeno común (Wakabayashi y col. 1984). Mas tarde, Baxa (1988) usando la técnica de aglutinación en portaobjetos con antisueros obtenidos con dos diferentes cepas de *T. maritimum*, estableció la existencia de seis serotipos dentro de la especie. Sin embargo, esta heterogeneidad serológica no fue confirmada por Pazos y col. (1993) cuando estudiaron los lipopolisacáridos (LPS) de 10 aislados españoles de *T. maritimum* usando la técnica de "immunoblot".

Estudios posteriores usando diversos métodos serológicos indican la existencia de diferentes grupos antigénicos dentro de *T. maritimum* (Pazos 1997; Ostland y col. 1999). Así, Pazos (1997) logró establecer mediante ensayos de "dot-blot" e "immunoblot" la existencia de a lo menos cuatro grupos serológicos entre aislados de rodaballo, salmón, pargo japonés, lubina, lenguado y las tres cepas de referencia. Éstas diferencias serológicas intra-específicas fueron discriminadas sólo cuando utilizó sueros absorbidos, y no pudo establecer relación entre el origen de las cepas de *T. maritimum* y el grupo serológico al que éstas pertenecían. La existencia de heterogeneidad serológica fue también demostrada por Ostland y col. (1999), al estudiar los aislados de salmón del Atlántico y las cepas de referencias mediante las técnicas de inmunodifusión en geles e "immunoblot". Sin embargo, la falta de concordancia en los grupos serológicos establecidos, probablemente debido a la diferencia en los antígenos, antisueros y técnicas empleadas, genera la necesidad de mayores estudios, para así poder establecer un claro esquema de serotipado dentro de *T. maritimum* (Capítulo III: artículos 1 y 2).

En cuanto al análisis de las proteínas de membrana, tanto totales como externas, se ha demostrado que todas las cepas de *T. maritimum* estudiadas, independiente de las características serológicas y la fuente de aislamiento presentan un perfil proteico similar. Así, Bernardet y col. (1994) definen a *T. maritimum* como una especie homogénea en cuanto a la composición proteica de la envoltura celular. Resultados similares han sido descritos por Pazos (1997), quien observó en los aislados de *T. maritimum* una gran cantidad de proteínas, con masas moleculares comprendidas entre 14,4 y 97,4 kDa. Sin embargo, pese a esta similitud proteica, el autor observó ligeras diferencias que estaban relacionadas con el grupo serológico y con el origen de los aislados, las que le hicieron definir a *T. maritimum* como una especie heterogénea antigénicamente en cuanto a la composición proteica de la envoltura celular.

Tabla 3. Principales características de la especie *Tenacibaculum maritimum* (Wakabayashi y col. 1986; Chen y col. 1995; Gosink y col. 1998; Suzuki y col. 2001; Avendaño-Herrera y col. 2004: artículo nº 1). + , prueba positiva; – , prueba negativa; d, resultado débil; O, oxidativa; [a] , resultados diferentes detectados en las descripciones publicadas por los diferentes investigadores.

Carácter	***Tenacibaculum maritimum***
Movilidad deslizante	+
Pigmento de flexirrubina	–
Adherencia al agar	(+)
Absorción de rojo congo	+
Gram	–
Metabolismo (O/F)	O
Reducción de nitratos	+ [a]
Tamaño celular (µm)	2 – 30 x 0,5
Morfología de la colonia:	
Forma	Plana, delgada y bordes irregulares
Tamaño 5 días de incubación	< 5 mm
Color	Amarillo pálido
Temperatura de (ºC):	
Crecimiento	15 – 34
Óptima	30
Rango de salinidad:	
NaCl	No crece
Agua de mar (%)	30 – 100
Rango de pH	5,9 – 8,6
Producción de:	
Catalasa	+
Citocromo oxidasa	+
H_2S	– [a]
Degradación de:	
Caseína	+
Gelatina	+ [a]
Almidón	–
Tirosina	+
Carboximetilcelulosa	–
Quitina	–
Crecimiento en:	
Casaminoácidos	+
N-acetilglucosamina	– [a]
Sucrosa	– [a]
D-ribosa	– [a]
DL-aspartato	–
L-prolina	–
L-glutamato	d (–)
Contenido de G+C (mol %)	31,3 – 32,5

1.3.4. Caracterización genética

A pesar de que la tenacibaculosis es una enfermedad relativamente antigua, son aún escasos los estudios encaminados a detectar diferencias a nivel genético entre cepas de su agente causal, *T. maritimum*. Los primeros estudios del genoma de *T. maritimum* realizados mediante la técnica de hibridización ADN-ADN, mostraron una gran relación entre las cepas evaluadas y no permitieron establecer heterogeneidad genética entre los aislados (Bernardet y Grimont 1989; Bernardet y col. 1994). En ambos estudios, las cepas procedentes de Europa y Japón mostraron un valor superior al 73-74% de relación con la cepa tipo de referencia (NCIMB 2154^{T}). Cabe señalar que este rango de homología es considerablemente menor al 92 a 100% descrito previamente por Baxa y col. (1987b) para aislados japoneses de *T. maritimum*, cuando utilizaron la técnica de análisis de secuencias polinucleotídicas por medición óptica de la re-naturalización del ADN.

Con posterioridad, Pazos (1997) al aplicar la técnica de ribotipado en 19 cepas del patógeno, incluyendo las cepas de referencias, demostró la existencia de diversidad intra-específica a nivel genómico dentro de la especie *T. maritimum*. Sin embargo, los patrones de ribotipado obtenidos dependerían directamente de la enzima seleccionada para la digestión del ADN y no pueden ser utilizados con fines epidemiológicos para la determinación del origen de los aislados. Por ello, se necesita una evaluación de otros métodos de genotipado que permitan el seguimiento de los clones bacterianos generadores en epizootias y que sean una herramienta valiosa para los estudios epidemiológicos de *T. maritimum* (Capítulo IV: artículo nº 3).

1.4. Factores de virulencia de *T. maritimum*

La mayoría de los estudios sobre mecanismos de virulencia de patógenos de peces han sido centrados particularmente en cepas específicas o en factores de virulencias puntuales, por lo que no es siempre fácil determinar su compromiso real en el desarrollo del progreso de la enfermedad (Toranzo y Barja 1993). En el caso particular de *T. maritimum*, pese a que es un patógeno conocido desde hace años, se han realizado pocos trabajos encaminados a esclarecer los factores asociados a su virulencia.

Por otro lado, como ha sido previamente señalado en el apartado de transmisión y reservorio (ver 1.2.2.), la mayoría de las infecciones experimentales han demostrado que *T. maritimum* provoca mortalidades variables y dependen directamente del método de infección empleado (aplicación tópica, inmersión, inoculación sub-dérmica e inyección intramuscular), lo que hace difícil la comparación de los resultados obtenidos en los diferentes laboratorios debido a la falta de uniformidad en las condiciones experimentales (preparación del inóculo, dosis de infección y temperatura) y en el tamaño y especie de pez utilizada.

Si bien, un número considerable de toxinas extracelulares y enzimas han sido asociadas con la virulencia de *T. maritimum* (Baxa y col. 1988b), existen otros factores que también podrían encontrarse en el patógeno, incluyendo adhesinas, citotoxinas, factores anti-fagocitosis, resistencias al efecto bactericida del complemento, habilidad de secuestrar hierro, capacidad de penetrar el epitelio celular, propiedades para sobrevivir y multiplicarse en los fagocitos o bien estructuras propias del microorganismo como capas proteicas adicionales, antígeno "O", fimbrias u otros mecanismos de adherencia no específicos como han sido descritos en diversos patógenos de peces (Dalsgaard 1993; Toranzo y Barja 1993). A continuación se expone el conocimiento existente en *T. maritimum* de algunos de los mecanismos señalados anteriormente.

1.4.1. Adherencia bacteriana

La adherencia de un patógeno al tejido epitelial del hospedador ha sido descrita como uno de los pasos iniciales de la patogénesis microbiana (Ofek y Doyle 1994). Así, la colonización a menudo requiere una firme adhesión de la bacteria a receptores existentes sobre las células del huésped por medio de moléculas de adhesinas específicas presentes en su superficie. En este proceso, la motilidad de la bacteria y las características hidrofóbicas parecen jugar un papel importante en la adhesión bacteriana.

Burchard y col. (1990) han observado que la capacidad de adhesión de las células de *T. maritimum* se incrementa significativamente en substratos con superficies que tienen un índice crítico de energía bajo, es decir, superficies hidrofóbicas. De esta forma, la bacteria produce una acumulación substancial de polímeros extracelulares o "slime" antes de adherirse, que va acompañado con un cambio en las propiedades superficiales de la célula y que favorecen la adhesión firme en substratos hidrofóbicos y más tenue en superficies hidrofílicas. Esta característica podría ser ventajosa para *T. maritimum*, ya que le podría permitir la supervivencia intracelular y la multiplicación en el tejido de los peces enfermos como ha sido descrito para otros microorganismos patógenos (Toranzo y Barja 1993), así como explicar porque células del patógeno se encuentran sistemáticamente en diferentes áreas del tejido externo del pez una vez que la infección se ha iniciado.

Por otro lado, estudios de adhesión "*in vitro*" han demostrado que las condiciones de crecimiento y disponibilidad de nutrientes provocan cambios considerables en la hidrofobicidad y adhesión de las células de *T. maritimum*, las cuales van acompañadas de cambios en las características de la superficie externa de *T. maritimum* (Sorongon y col. 1991). Así por ejemplo, en condiciones de cultivo estáticas *T. maritimum* incrementa considerablemente la producción de diversos polipéptidos en la superficie externa de la célula, las cuales son mucho mayores a las originadas cuando el microorganismo es crecido en medio sólido o medio líquido sin agitación. Asimismo, Carson y col. (1993) han demostrado que cuando las cepas de *T. maritimum* crecían en medio de cultivo preparado con agua de mar artificial eran menos adherentes que al cultivarlas con agua de mar natural, y además perdían su virulencia. La presencia de otros polímeros no detectados por Sorongon y col. (1991) no deben ser ignorados, entre ellos, LPS y polisacáridos extracelulares, así como características constitutivas propias de la bacteria como por ejemplo la absorción del colorante rojo Congo, que indica la producción del glucano galactosamina y que interesantemente permite diferenciar cepas virulentas de aquellas no virulentas en la bacteria patógena de peces *A. salmonicida* (Ishiguro y col. 1985).

Aunque Pazos (1997) mediante técnicas de aglutinación con distintos tipos de eritrocitos demostró que *T. maritimum* posee propiedades hemaglutinantes, las cuales serían reguladas por la presencia de diferentes adhesinas en la superficie celular de la bacteria, el mismo autor señala que no existe relación de los patrones observados con el origen de los aislados o la virulencia de los mismos. Asimismo, en el estudio no se determinó el comportamiento de las adhesinas en el mecanismo de infección del patógeno.

Otros componentes implicados en los mecanismos de adherencia de diversos microorganismos patógenos están relacionados con la presencia de estructuras como pili y/o fimbrias y material capsular (Toranzo y Barja 1993; Toranzo y col. 2005). Hasta ahora se desconocía la existencia de algunos de estos componentes celulares en el patógeno, en esta memoria se demuestra la presencia de cápsula en *T. maritimum* (Capítulo VII).

Sin embargo, la adherencia del patógeno a los tejidos del hospedador depende directamente de la capacidad de neutralizar los mecanismos de defensa del pez, entre ellos la actividad bactericida del mucus. Magariños y col. (1995) han demostrado que *T. maritimum* posee una gran capacidad de adherencia al mucus de los peces, así como la resistencia a la acción bactericida de este material mucoso, y por tanto, la superficie del pez constituye una puerta de entrada muy importante para el patógeno. Aunque, la bacteria es considerablemente susceptible a la actividad antibacteriana de otros microorganismos que comparten su nicho ecológico, incluyendo especies del género *Vibrio*, *Pseudomonas* y *Alteromonas* (Pazos y col. 1996; Avendaño-Herrera y col. 2005: artículo nº 6), el mucus de los peces actuaría como una película protectora para *T. maritimum* frente a las bacterias antagonistas.

1.4.2. Producción de exo- y endotoxinas

Las exotoxinas son proteínas extracelulares sensibles al calor y que se encuentran divididas en dos tipos: a) aquellas que actúan extracelularmente o sobre membranas celulares, atacando substancias intercelulares o superficies celulares a través de mecanismos enzimáticos o de tipo detergentes, incluyendo hemolisinas bacterianas, leucocidinas, colagenasas, fribrinolisinas, fosfolipasas e hialuronidasas, las cuales juegan un papel importante en las infecciones; y b) aquellas proteínas o polipéptidos que entran en la célula y enzimáticamente alteran los procesos celulares.

Como *T. maritimum* se aísla a partir de lesiones externas se ha sugerido que exotoxinas pertenecientes al primer grupo pueden estar implicadas como primer mecanismo de virulencia y probablemente ayudando a la colonización de los tejidos del pez. Sin embargo, Campbell y Buswell (1982) describieron que la inoculación de lenguados con los sobrenadantes de cultivos de cepas de *T. maritimum* no produjeron efecto alguno en los peces, lo cual podría ser explicado sobre la base de que es la invasión de los tejidos por parte de la bacteria, y no la producción de toxinas, lo que causa las mortalidades.

Estos resultados discrepan con los estudios realizados por Baxa y col. (1988b), quienes al analizar las actividades "*in vivo*" de preparados de exotoxinas producidas por una cepa aislada de seriola, observaron que los ECP y las hemolisinas de *T. maritimum* causaban una notable mortalidad cuando eran inyectados en peces por vía intraperitoneal. De esta forma, concluyen que la patogenicidad de *T. maritimum* puede ser atribuída a la interacción sinérgica de las toxinas y enzimas presentes en los ECP. Mas tarde, Pazos (1997) al estudiar la composición de los ECP de 19 cepas de *T. maritimum* determinó que éstos contienen niveles elevados de proteínas y LPS, los cuales poseían en una alta actividad proteolítica y contenían diversas enzimas, incluyendo gelatinasas, caseinasas, amilasas, ADNasas y hemolisinas. Paralelamente, también observó la presencia de actividad citotóxica en los ECP de todos los aislados estudiados usando líneas celulares de peces.

Con respecto a las endotoxinas o LPS, se conoce que son una de las toxinas más importantes presentes en las bacterias Gram negativas, siendo probablemente responsables directas de algunos de los signos característicos durante una infección originadas por este grupo de microorganismos (Dalsgaard 1993). Estos componentes son extremadamente sensibles al calor comparadas con las exotoxinas, y se encuentran formadas por cadenas laterales de polisacáridos (cadena “O”), un núcleo polisacárido o “core” y el lípido A, que es el componente que confiere toxicidad al complejo LPS. Dentro de las propiedades de las endotoxinas es su actividad antigénica, que está asociada al antígeno somático termoestable “O”, la cual le puede proveer de su especificidad serológica como ha sido puesta de manifiesto en esta memoria (Avendaño-Herrera y col. 2004; artículos 1 y 2).

Baxa y col. (1988b) al estudiar la patogenicidad en pargo y besugo japonés de los componentes brutos o puros de los LPS obtenidos de una cepa de *T. maritimum*, no observaron mortalidad en ninguno de los grupos de peces inoculados por vía intraperitoneal, pero si la detectaron cuando utilizaron simultáneamente los LPS brutos y proteasas. Por otro lado, los peces supervivientes a la inoculación con LPS mostraron signos claros de la infección, incluyendo exoftalmia y tejido hemorrágico, mientras que las exotoxinas no provocaron ningún síntoma en los peces. Es interesante mencionar que actividad de tipo lipasa fue detectada en los LPS, lo que sugiere que esta actividad podría permanecer asociadas a la superficie celular y no ser secretada al medio.

Vinogradov y col. (2003) han estudiado la estructura de la cadena lateral del polisacárido del LPS de una cepa de *T. maritimum*, encontrando por primera vez la presencia de un residuo "(*R*)-2-hydroxyglutaric acid" en un polisacárido bacteriano. Aunque un componente similar había sido previamente descrito en la estructura del polisacárido "O" en el patógeno de peces *Flavobacterium psychrophilum* (MacLean y col. 2001), ambas estructuras son completamente diferentes desde el punto de vista serológico. Si bien la producción del polisacárido "O" probablemente contribuye a las propiedades de la superficie bacteriana y su capacidad de formar biopelículas, consideramos que son necesarios mayores estudios y que incluyan un número mayor de aislados de *T. maritimum*.

En conclusión, las proteasas, hemolisinas y citotosinas detectados en los ECP de *T. maritimum* junto con los LPS son factores tóxicos que pueden explicar las hemorragias, histolisis y necrosis que son tan característicos en los peces afectados por tenacibaculosis, así como en infecciones originadas por otras bacterias filamentosas (Dalsgaard 1993).

1.4.3. Mecanismo de captación de hierro y contenido plasmídico

Otro mecanismo de virulencia que influye decisivamente en el proceso de infección de algunos patógenos bacterianos de origen marino es la presencia de un sistema de captación de hierro (Fe), que le permitiría a la bacteria crecer y multiplicarse en el interior del hospedador. Puesto que todo el Fe disponible en el cuerpo del huésped se halla fuertemente unido a proteínas de defensa como hemoglobina, mioglobina, ferritina, hemosiderina, transferrina, lactoferrina, entre otras, la bacteria necesita de mecanismos que le permita la asimilación del Fe a partir de las proteínas señaladas, o a partir de los grupos hemo libres (ver revisión Neilands 1982; Ratledge y Dover. 2000).

Uno de los mecanismos de obtención de Fe implica la interacción directa entre receptores de la superficie celular bacteriana y las ferro-proteínas. Aunque el mecanismo mejor conocido hasta la fecha y por el cual las bacterias patógenas obtienen el Fe, es aquél que implica la producción de sustancias quelantes extracelulares, denominadas sideróforos, ello no significa que sea el más importante en el suministro de Fe, sino que simplemente puede ser más fácilmente abordable su estudio (ver revisión Neilands 1982; Ratledge y Dover. 2000). Estos mecanismos de adquisición de hierro han sido vinculados a la virulencia en algunos patógenos de peces como *L. anguillarum* (Wolf y Crosa 1986; Muiño y col. 2001), *A. salmonicida* (Ishiguro y col. 1986; Hirst y col. 1991), *Ph. damselae* subsp. *piscicida* (Magariños y col. 1994; do Vale y col. 2002) y *Edwardsiella tarda* (Iida y Wakabayashi 1990; Igarashi y col. 2002). Como se describirá en esta memoria, *T. maritimum* posee también mecanismos de captación de Fe, mediados y no por sideróforos (Capítulo VII: artículo 7), que podrían contribuir a su patogenicidad.

En algunos patógenos marinos ha sido claramente demostrado que la presencia de un sistema de transporte de hierro esta fuertemente relacionada con la existencia de contenido extracromosomal (ver revisión Toranzo y Barja 1993). El caso más conocido de este mecanismo mediado por genes extracromosómico ha sido descrito por Crosa (1980), quien demostró que la cepa salvaje de *L. anguillarum* 775 podía crecer fácilmente en presencia de transferrina, mientras que mutantes de esta cepa se veían inhibidas en presencia de esta proteína. Esta propiedad estaba asociada a la presencia de un plásmido de 65 Kilobases (Kb) denominado pJM1, que está presente en la mayoría de las cepas pertenecientes al serotipo O1 (Tolmasky y col. 1985; Toranzo y Barja 1990; Conchas y col. 1991) y que participa en la producción del sideróforo de tipo fenolato llamado anguibactina (Actis y col. 1986), el cual es un importante factor en la virulencia de *L. anguillarum*.

Aunque en los estudios preliminares de Pazos (1997) no se observó la presencia de contenido plasmídico en cepas de *T. maritimum*, en esta memoria se ha analizado en profundidad la posible existencia de material extracromosomal empleando diferentes técnicas (Capítulo VII).

1.5. Tratamiento y prevención de la tenacibaculosis

1.5.1. Quimioterapia

Los estudios centrados en la susceptibilidad "*in vitro*" de *T. maritimum* a agentes quimioterápicos demuestran que este microorganismo, independiente del huésped de aislamiento y origen geográfico exhibe un patrón similar, siendo susceptible a nitrofuranos, penicilinas (ampicilina y penicilina G), eritromicina, tetraciclinas (tetraciclina y oxitetraciclina), cloranfenicol, trimetoprin, sulfamidas potenciadas y quinolonas fluoradas (enrofloxacina y flumequina) (Baxa y col. 1988c; Alsina y Blanch 1993; Pazos y col. 1993; Chen y col. 1995; Soltani y col. 1995; Avendaño-Herrera y col. 2004; 2005: Capítulos III y VIII: artículos nº 1 y 8) y resistentes a ácido oxolínico, clortetraciclina, colistina, aminocidina, bacitracina, kanamicina, neomicina y estreptomicina, aunque este último agente quimioterápico puede tener una respuesta variable (Baxa y col. 1988c; Alsina y Blanch 1993; Pazos y col. 1993; Cepeda y Santos 2002).

En general, como *T. maritimum* se aísla con más frecuencia a partir de la superficie externa de los peces, podría pensarse que el uso de agentes quimioterápicos administrados por inmersión o baños serían más eficientes para el control de la tenacibaculosis que el tratamiento por pienso medicado, ya que una vez que la infección comienza los peces disminuyen su apetito, lo que puede provocar un estado anoréxico en los peces (Campbell y Buswell 1982; Baxa y col. 1988c; Soltani y col. 1995). Sin embargo, los trabajos orientados al tratamiento de la tenacibaculosis aún son escasos y con resultados muy variables.

Los primeros antecedentes sobre el control de la tenacibaculosis con quimioterápicos son señalados por McVicar y White (1979), quienes al usar una mezcla de antibióticos de amplio espectro ("Furanace®", terramicina, kanamicina, "Tribrissen®" y "Tylan®") en una concentración de 30 – 50 mg ingrediente activo por kg de pez por día por vía oral para el tratamiento de lenguados infectados no observaron ninguna disminución en las mortalidades de los peces. Por el contrario, baños con penicilina G (17,5 g m^{-3}) o con sulfato de estreptomicina (66 g m^{-3}) fueron efectivos en el control de la tenacibaculosis, pero sólo a corto plazo. Similares resultados han sido señalados por Bernardet y col. (1994), quienes denotan que el uso rutinario de nitrofurano por baño para el tratamiento de lubinas infectadas con *T. maritimum* en Francia muestran pobres resultados. Probablemente, el uso continuado de un mismo agente quimioterápico en las infecciones de los peces provoque la aparición de cepas bacterianas resistentes a dichos compuestos como ha sido demostrado en otros patógenos de peces (Smith y col. 1994; Alderman y Hastings 1998) y también recientemente en *T. maritimum* (Capítulo VIII: artículo nº 9).

Alsina y Blanch (1993) indican que la furazolidona es un tratamiento efectivo para detener las mortalidades en juveniles de rodaballo, y señalan la existencia de una buena correspondencia con los resultados de susceptibilidad obtenidos en los antibiogramas. Es un hecho conocido que los resultados del tratamiento de los peces con quimioterápicos no siempre son satisfactorios, aún a pesar de que las bacterias aisladas sean, "*in vitro*", altamente susceptibles a los compuestos.

Hasta la fecha, sólo Soltani y col. (1995) han estudiado "*in vitro*" e "*in vivo*" la eficacia de algunos quimioterápicos en *T. maritimum*, observando una óptima correlación entre las dos respuestas cuando usaron amoxicilina y trimetoprin. Estos quimioterápicos presentaron niveles adecuados en el suero de salmón del Atlántico y trucha arcoiris, tanto cuando fueron administrados por vía oral, así como por baño. Cabe destacar que aunque la amoxicilina es clínicamente más eficaz en el control de la tenacibaculosis a corto plazo, el trimetoprin es a menudo más protectivo a largo plazo (Soltani y col. 1995; Handlinger y col. 1997), y esto puede ser debido a que este compuesto se acumula en la piel del pez y es muy persistente, combatiendo la infección superficial y la sistémica, particularmente en salmónidos (Cameron 1993; Carson y col. 1992).

Otro tratamiento comúnmente usado para controlar la tenacibaculosis en salmónidos cultivados en Australia es la oxitetraciclina (75 mg por kg de pez por día), proporcionando una satisfactoria respuesta de los peces (Handlinger y col. 1997). En Europa, a partir del año 2000, el quimioterápico más frecuentemente usado en la gran mayoría de las piscifactorías de la península Ibérica ha sido la enrofloxacina. Este quimioterápico inicialmente actúa de forma efectiva en el control de la enfermedad, pero su uso rutinario ha dado lugar a la aparición de cepas bacterianas resistentes a dicho compuesto (Capítulo VIII: artículo 9).

1.5.2 Desinfección y otras medidas profilácticas alternativas

Considerando los inconvenientes derivados de la utilización irracional de los quimioterápicos (tales como la selección de cepas resistentes y el impacto ambiental provocado por la concentración de estos compuestos en los sedimentos y fauna próxima a las piscifactorías), el uso de desinfectantes externos como formol podría constituir un método alternativo para el control de la tenacibaculosis. Sin embargo, McVicar y White (1979) señalan que el tratamiento rutinario y continuo con formol (40 ppm), si bien, ayuda a controlar la enfermedad, no la elimina completamente y tiene el inconveniente de que su uso prolongado causa daño en las branquias de los peces. Del mismo modo, el uso de permanganato de potasio ha sido sugerido para el tratamiento del agua de cultivo de lubinas en brotes ocurridos en Malta (Bernardet 1998), pero no existen estudios sobre el efecto de este compuesto en los peces.

El peróxido de hidrógeno (H_2O_2), es otro tratamiento profiláctico que ha recibido una gran atención por su efectivo control de diversos patógenos externos de peces, particularmente aquellos del género *Flavobacterium* y *Cytophaga* (Lumsden y col. 1998; Derksen y col. 1999; Thomas-Jinu y Goodwin, 2004), que son fenotípicamente similares a *T. maritimum*. Aunque no existían estudios sobre el uso de este compuesto sobre *T. maritimum*, en esta memoria ha sido puesto de manifiesto su efecto sobre el patógeno y sobre los peces infectados (Capítulo VIII: artículo 10).

El control de la temperatura y/o salinidad puede ser empleada para disminuir la morbilidad que *T. maritimum* produce en salmónidos, siendo recomendado reducir la temperatura a menos de 15ºC y mantener la salinidad por debajo de 10 g l^{-1} para prevenir o controlar las erosiones cutáneas producidas por el microorganismo (Soltani y Burke 1994). Sin embargo, el control de la enfermedad mediante la modificación de los factores abióticos del agua de cultivo presupone importantes dificultades técnicas e inversiones de capital en los cultivos a gran escala, así como el inconveniente de que también reducen la tasa de crecimiento de los peces. Al mismo tiempo, estas manipulaciones de la temperatura y salinidad pueden producir un estrés en los peces (variable dependiendo de la especie), lo que puede incrementar la susceptibilidad a otras enfermedades causadas por diversos patógenos.

Por otro lado, en lenguados, McVicar y White (1979; 1982) consiguieron evitar y disminuir las tasas de mortalidad provocadas por *T. maritimum* por medio de la inclusión de una capa de arena de 0,5 – 0,75 cm en el fondo de los tanques. Según los mismos autores, este substrato causa una considerable reducción en los niveles de estrés y permiten mantener una comportamiento natural de enterramiento en los lenguados, lo que podría ayudar a cuidar la cutícula y favorecer un óptimo estado en la epidermis de los peces. Esta introducción de arena requiere también de grandes dificultades técnicas entre ellas, evitar la perdida de arena, mantener una óptima densidad de peces, prevenir las condiciones anaeróbicas y exceso de alimentación, así como una rigurosa limpieza de los tanques (McVicar y White 1982).

En conclusión, la incidencia de brotes de la tenacibaculosis puede al menos reducirse mediante una correcta manipulación de los peces, el control de sus densidades, la regulación de la alimentación y una higiene adecuada de las instalaciones.

1.5.3. Vacunación

Hoy por hoy, la vacunación es la única medida que permite evitar la aparición de enfermedades de origen bacteriano en las granjas de cultivo de peces en todo el mundo. Håstein y col. (2005) señalan que existen formulaciones vacunales contra al menos 15 microorganismos patógenos de peces en todo el mundo, la mayoría de ellas son usadas con el respectivo permiso de los países en los cuales son utilizadas, siendo administradas preferentemente a los peces por inyección, inmersión u oral. Teniendo en cuenta que la mayoría de las epizootias de tenacibaculosis han sido descritas en rodaballo y salmónidos cultivados en España y Australia respectivamente, es coherente que sólo en estos dos países se hallan desarrollado vacunas contra la enfermedad (Håstein y col. 2005).

En el caso particular del cultivo de rodaballo en España, la tenacibaculosis ha sido un enorme problema entre 1996 a 1999 (Toranzo y col. 2004), lo que generó la necesidad de desarrollar un programa de vacunación. Así nuestro grupo de investigación ha evaluado una vacuna específica patentada por la Universidad de Santiago de Compostela para la prevención de la tenacibaculosis marina en el rodaballo (FM-95) (Santos y col. 1999). La vacuna es administrada por inyección a peces mayores de 40 g y confiere niveles eficaces de protección de hasta 85%. Además y puesto que la enfermedad puede afectar a las fases larvarias de rodaballo, se aconseja la administración de dos dosis vacunales vía baño, separadas por un intervalo de 4 semanas, seguidas de una tercera dosis intraperitoneal cuando los peces alcanzan un peso de 20 – 30 g (Romalde y col. 2005; Toranzo y col. 2005). El uso de esta bacterina en determinadas granjas ha provocado una disminución de la incidencia de la tenacibaculosis en rodaballo en los últimos años (Toranzo y col. 2004). Sin embargo, podría no ser una medida eficaz para controlar la enfermedad en otras especies, como lenguado y dorada, siendo necesario el desarrollo de nuevos programas de vacunación. De hecho, nuestro grupo de investigación está evaluando una nueva bacterina específica para la prevención de la tenacibaculosis marina en lenguados (*S. senegalensis*). Los estudios realizados mediante infecciones experimentales han mostrado un porcentaje de supervivencia de 94% cuando la vacuna es administrada a los peces intraperitonealmente (Romalde y col. 2005; Toranzo y col. 2005)

Por el contrario, los estudios publicados sobre vacunación en Australia son escasos, y sólo hemos encontrado los estudios realizados por Carson y col. (1993), quienes al ensayar en salmónidos diferentes formulaciones vacunales, obtuvieron niveles de protección que oscilan entre el 21 y 70%.

La composición de la mayoría de las vacunas contra la tenacibaculosis usan células enteras inactivadas mediante el tratamiento con formol o calor. Estudios recientes de Salati y col. (2005) han demostrado que como en la mayoría de los patógenos, el componente inmunogénico principal de las vacunas frente a *T. maritimum* son los LPS.

II. OBJETIVOS

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La trascendencia económica que provoca la aparición de *Tenacibaculum maritimum* en los cultivos intensivos de distintas especies de peces marinos en todo el mundo, ha incrementado nuestro interés por profundizar en el estudio de este microorganismo. A pesar que la tenacibaculosis es una enfermedad conocida desde hace muchos años, no han sido realizados los esfuerzos suficientes para el conocimiento de su agente causal en comparación con otros patógenos de peces. Por ello, en esta memoria se pretende aclarar diversos aspectos que ayuden al desarrollo de medidas que permitan un rápido diagnóstico de la enfermedad, así como su prevención y control.

Por lo tanto, nos hemos propuestos los siguientes objetivos:

1. Caracterizar a nivel antigénico los aislados de *T. maritimum*, con el fin de definir un esquema claro de serotipado y así poder determinar la posible existencia de serotipos predominantes asociados a las mortalidades.
2. Analizar la existencia de variabilidad genética intra-especifica mediante análisis de polimorfismo genómico (RAPD), con el objeto de evaluar la utilidad de esta técnica como herramienta en estudios epidemiológicos.
3. Comparar la especificidad y sensibilidad de los protocolos de PCR descritos para la identificación de *T. maritimum* en ensayos "*in vitro*" y estudios de campo, con la finalidad de desarrollar un protocolo de PCR no letal, altamente sensible, específico y de fácil aplicación que permita la rápida detección de *T. maritimum* tanto en peces sintomáticos como portadores de la enfermedad.
4. Estudiar la supervivencia de *T. maritimum* en el agua de mar, con el fin de determinar el papel del medio acuático en la epidemiología de las infecciones producidas por *T. maritimum*, así como el posible papel de la microbiota autóctona del agua en la viabilidad de esta bacteria.

5. Estudiar los mecanismos de captación del hierro y el papel que pueden jugar en la expresión de la virulencia del patógeno.

6. Evaluar la eficacia de diferentes compuestos antibacterianos para el posible control de la tenacibaculosis.

Para llevar a cabo estos objetivos, utilizamos las siguientes cepas de *Tenacibaculum maritimum* que incluyen aislados de epizootias en diferentes países y cepas de referencias:

Cepa	**Origen**	**País**	**Año de Aislamiento**
LR2P	Lenguado(*Solea solea*)	España	1995
PC477.1	Lenguado (*Solea senegalensis*)	España	2001
PC477.4	(*Solea senegalensis*)	España	2001
PC487.1	(*Solea senegalensis*)	España	2001
PC492.1	(*Solea senegalensis*)	España	2001
PC503.1	(*Solea senegalensis*)	España	2001
PC504.1	(*Solea senegalensis*)	España	2001
PC528.1	(*Solea senegalensis*)	España	2002
PC529.1	(*Solea senegalensis*)	España	2002
PC517.1	(*Solea senegalensis*)	España	2002
PC432.1	(*Solea senegalensis*)	España	2002
PC500.1	(*Solea senegalensis*)	España	2002
AZ202.1	(*Solea senegalensis*)	España	2001
AZ203.1	(*Solea senegalensis*)	España	2001
IEO8.1	(*Solea senegalensis*)	España	2003
LgH35-03 *	(*Solea senegalensis*)	España	2003
LgV1-04 *	(*Solea senegalensis*)	España	2004
ACC6.1	(*Solea senegalensis*)	Portugal	2003
ACC8.1	(*Solea senegalensis*)	Portugal	2003
ACC13.1	(*Solea senegalensis*)	Portugal	2003
ACC20.1	(*Solea senegalensis*)	Portugal	2004
ACC21.1	(*Solea senegalensis*)	Portugal	2004
ACC22.1	(*Solea senegalensis*)	Portugal	2004
ACC23.1	(*Solea senegalensis*)	Portugal	2004
ACC27.1	(*Solea senegalensis*)	Portugal	2004
ACC28.1	(*Solea senegalensis*)	Portugal	2004
PC424.1	Rodaballo (*Scophthalmus maximus*)	España	2000
PC460.1	(*Scophthalmus maximus*)	España	2001
PC473.1	(*Scophthalmus maximus*)	España	2001
PC394.1	(*Scophthalmus maximus*)	España	2000
LD12.1	(*Scophthalmus maximus*)	España	2001
RM256.1	(*Scophthalmus maximus*)	España	2002
LPV1.7	(*Scophthalmus maximus*)	España	1995
RI93.1	(*Scophthalmus maximus*)	España	2002
ACR104.1	(*Scophthalmus maximus*)	España	2001
RM268.1	(*Scophthalmus maximus*)	España	2002
RM261.1	(*Scophthalmus maximus*)	España	2002
RM276.1	(*Scophthalmus maximus*)	España	2004
LD29.1	(*Scophthalmus maximus*)	España	2004
PC682.1	(*Scophthalmus maximus*)	España	2003
JIP 24/99 **	(*Scophthalmus maximus*)	España	1999
JIP 46/00 **	(*Scophthalmus maximus*)	España	2000

Cepa	**Origen**	**País**	**Año de Aislamiento**
PC538.1	Dorada (*Sparus aurata*)	España	2002
PC560.1	(*Sparus aurata*)	España	2002
DOB102	(*Sparus aurata*)	España	2002
342101	(*Sparus aurata*)	España	2001
PC824.1	(*Sparus aurata*)	España	2003
PC868.1	(*Sparus aurata*)	España	2003
SSG33	Salmón del Atlántico (*Salmo salar*)	España	1993
SO127	(*Salmo salar*)	España	1993
JIP 32/99 **	Lubina (*Dicentrarchus labrax*)	Francia	1999
LVDH 1577.01 **	Lubina (*Dicentrarchus labrax*)	Francia	2003
DBA4a **	Seriola (*Seriola quinqueradiata*)	Japón	1986
NCIMB 2158	Lenguado (*Solea solea*)	Escocia	1981
NCIMB 2153	Besugo (*Acanthopagrus schlegeli*)	Japón	1976
NCIMB 2154^T	Pargo Japonés (*Pagrus major*)	Japón	1977

NCIMB, Colección Nacional de Bacterias Industriales y Marinas (Aberdeen, Escocia).

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III. CARACTERIZACIÓN BIOQUÍMICA Y SEROLÓGICA DE LAS CEPAS DE *Tenacibaculum maritimum*

III.A. PUBLICACIONES

Artículo nº 1: Phenotypic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains from marine fish. **Diseases of Aquatic Organisms (2004). 58:** 1-8.

Artículo nº 2: A novel O-serotype in *Tenacibaculum maritimum* strains isolated from cultured sole (*Solea senegalensis*). **Bulletin of the European Association of Fish Pathologists (2005). 25**: 70-74.

CONTENIDO:

- Caracterización fisiológica y bioquímica de las cepas.
- Sensibilidad a antibióticos y agentes quimioterápicos.
- Estudio serológico.
- Análisis de LPS y proteínas de membrana.

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Phenotyphic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains from marine fishes

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ABSTRACT: *Tenacibaculum maritimum* is the etiological agent of marine flexibacteriosis disease, with the potential to cause severe mortalities in various cultured marine fishes. The development of effective preventive measures (i.e. vaccination) requires biochemical, serological and genetic knowledge of the pathogen. With this aim, the biochemical and antigenic characteristics of *T. maritimum* strains isolated from sole, turbot and gilthead sea bream were analysed. Rabbit antisera were prepared against sole and turbot strains to examine the antigenic relationships between the 29 isolates and 3 reference strains. The results of the slide agglutination test, dot-blot assay and immunoblotting of lipopolysaccharides (LPS) and membrane proteins were evaluated. All bacteria studied were biochemically identical to the *T. maritimum* reference strains. The slide agglutination assays using O-antigens revealed cross-reaction for all strains regardless of the host species and serum employed. However, when the dot-blot assays were performed, the existence of antigenic heterogeneity was demonstrated. This heterogeneity was supported by immunoblot analysis of the LPS, which clearly revealed 2 major serological groups that were distinguishable without the use of absorbed antiserum: Serotypes O1 and O2. These 2 serotypes seem to be host-specfic. In addition, 2 sole isolates and the Japanese reference strains displayed cross-reaction with both sera in all serological assays, and are considered to constitute a minor serotype, O1/O2. Analysis of total and outer membrane proteins revealed that all strains share a considerable number of common bands that are antigenically related.

KEY WORDS: *Tenacibaculum maritimum* · O-antigen · Lipopolysaccharides · Serotype · Marine fishes

INTRODUCTION

Marine flexibacteriosis caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (Suzuki et al. 2001) constitutes one of the main problems in the aquaculture fishes. The disease is widely distributed in Japan, Europe and North America, and causes serious mortalities and hence severe economic losses in cultured marine fishes (Hikida et al. 1979, McVicar & White 1979, Wakabayashi et al. 1986, Devesa et al. 1989, Bernardet et al. 1990, 1994, Pazos et al. 1993, Chen et al. 1995, Ostland et al. 1999). In Europe, the presence of this pathogen was first demonstrated in Scotland for Dover sole *Solea solea* suffering 'black patch necrosis' (Bernardet et al. 1990). However, during the last decade, the development of the intensive commercial culture of fishes such as turbot *Scophthalmus maximus*, sole *Solea solea* and *S. senegalensis*, gilthead sea bream *Sparus aurata*, and sea bass *Dicentrarchus labrax* has caused an increased prevalence and severity of the disease.

The infection route of this microorganism is by direct attack on the body surface of fishes (Magariños et al. 1995), causing lesions such as ulcers, necrosis, eroded mouths, frayed fins and tail-rot (Campbell & Buswell 1982, Devesa et al. 1989). As these lesions favor the

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*Corresponding author. Email: mpaetjlb@usc.es entrance of other pathogenic bacteria such as *Vibrio* spp. (Kimura & Kusuda 1983) and saprophytic organisms such as ciliated protozoans (McVicar & White 1979, Devesa et al. 1989), *Tenacibaculum maritimum* thus often appears in mixed infections.

From a microbiological point of view, one of the major constraints on detection of *Tenacibaculum maritimum* is the lack of methods to distinguish this microorganism from others that are phenotypically similar, particularly from other marine flavobacteria (Suzuki et al. 2001). In addition, obtaining pure cultures of *T. maritimum* from external lesions is difficult, because of the characteristic slow growth of this pathogen which allows other opportunistic species to overgrow it. However, polymerase chain reaction (PCR) methodology has proved a powerful tool for accurately identifying the pathogen from plate cultures as well as from fish tissues (Toyama et al. 1996, Bader & Shotts 1998).

Numerous studies report that *Tenacibaculum maritimum* constitutes a biochemically, serologically and molecularly homogeneous taxon (Bernardet & Grimont 1989, Bernardet et al. 1990). However, recent studies using serological and molecular typing methods have demonstrated the existence of distinct serological subgroups within this species (Pazos 1997, Ostland et al. 1999, Santos et al. 1999), but there was no consensus among the serological groups established by the different authors, perhaps due to differences in the antigens, antisera and techniques used. The discrepancies underline the necessity for major studies, examining the association of the different antigenic subgroups with the original hosts and/or the geographic distribution of the bacterium. Detailed antigenic knowledge of this bacterial pathogen is of crucial importance not only from an epidemiological standpoint, but also to enable the development and formulation of appropriate and effective vaccine(s) (Romalde et al. 2003).

Therefore, the objective of this study was to determine in detail the biochemical and antigenic characteristics of *Tenacibaculum maritimum* isolates from different species of marine fishes, for future development of and/or improving vaccination programs against marine flexibacteriosis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. We isolated 29 *Tenacibaculum maritimum* strains from sole *Solea senegalensis* and *S. solea*, turbot *Scophthalmus maximus*, and gilthead sea bream *Sparus aurata* from 1995 to 2003 for use in the present study (Table 1). The bacterial strains were confirmed as *T. maritimum* by PCR-based analysis, as described by Toyama et al. (1996). *T. maritimum* Reference Strains NCIMB 2153, 2154^T and 2158 from the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) were used for comparative purposes. The bacteria were routinely cultivated on/in *Flexibacter maritimus* medium (FMM) agar or broth (Pazos et al. 1996) and incubated at 20°C for 72 h. Stock cultures were maintained frozen at –70°C in Criobille tubes (AES Laboratory, France).

Biochemical and genetic characterization. The strains were examined using phenotypic tests, basically as reported by Bernardet et al. (1990) and Ostland et al. (1999). Colony morphology and pigmentation, cell morphology, gliding motility, Gram-staining, 1% glucose oxidation or fermentation, cytochrome oxidase activity (*n*,*n*,*n*′,*n*′-tetramethyl-p-phenylenediamine, Sigma), catalase production on glass slides (using 3% H_2O_2), and the production of hydrogen sulfide, H_2S (Blei II-Acetatpapier, Merck) were determined. The presence of cell wall-associated flexirubin-type pigments and galactosamine glycans was determined by adding 20% KOH or 0.01% Congo red (Sigma), respectively, to 3 d-old colonies. Hydrolysis of the following substrates was determined using FMM as basal medium: starch (1%), Tween 80 (1 %), gelatin (2%), carboxymethylcellulose (3% CMC, Sigma), agar (1.5%), casein (2%), lecithin (5% sterile egg-yolk suspension) and chitin (20%). All isolates were tested for nitrate reduction (0.1% KNO_3) and esculin production (0.1% esculin). All tests were incubated aerobically for 7 d at 25°C.

To detect the presence and levels of enzymatic activity, each strain of *Tenacibaculum maritimum* was examined with the API ZYM (bioMérieux) miniaturized system according to the manufacturer's instructions, with the exception of the incubation temperature, which was fixed at 25°C.

The pattern of antimicrobial sensitivity was evaluated by the disc diffusion method on FMM agar using the following chemotherapeutic agents (Oxoid): ampicillin (10 µg), oxytetracycline (30 µg), chloramphenicol (30 µg), nitrofurantoine (300 µg), flumequine (30 µg), sulphamethoxazole/trimethoprim (25 µg), enrofloxacine (5 µg) and oxolinic acid (2 µg). Antibiogram readings were performed after 48 h incubation at 25°C.

Serological characterization. Serological assays were carried out using O-antigens of each strain obtained after heat-killing the bacterial suspensions (10^9 cells ml^{-1}) in phosphate-buffered saline, PBS (pH 7.4) at 100°C for 60 min, washing once in the same saline solution, and maintaining at 4°C until required.

Antisera against 2 strains isolated from sole and turbot, coded as PC503.1 and PC424.1 respectively, were prepared by intravenous injections of rabbits with formalin-killed cells (10^9 cells ml^{-1}) suspended in PBS

Table 1. *Tenacibaculum maritimum* strains used in this study. NCIB: National Collection of Industrial and Marine Bacteria (Aberdeen, UK); ATCC: American Type Culture Collection (Rockville, USA)

Bacterial isolate	Host species	Origin	Year of isolation
LR2P	Sole *Solea solea*	Spain	1995
PC477.1	Sole *Solea senegalensis*	Spain	2001
PC487.1	Sole *S. senegalensis*	Spain	2001
PC492.1	Sole *S. senegalensis*	Spain	2001
PC503.1	Sole *S. senegalensis*	Spain	2001
PC504.1	Sole *S. senegalensis*	Spain	2001
PC528.1	Sole *S. senegalensis*	Spain	2002
PC529.1	Sole *S. senegalensis*	Spain	2002
PC517.1	Sole *S. senegalensis*	Spain	2002
PC432.1	Sole *S. senegalensis*	Spain	2002
PC500.1	Sole *S. senegalensis*	Spain	2002
AZ202.1	Sole *S. senegalensis*	Spain	2001
AZ203.1	Sole *S. senegalensis*	Spain	2001
IEO8.1	Sole *S. senegalensis*	Spain	2003
PC424.1	Turbot *Scophthalmus maximus*	Spain	2000
PC460.1	Turbot *S. maximus*	Spain	2001
PC473.1	Turbot *S. maximus*	Spain	2001
PC394.1	Turbot *S. maximus*	Spain	2000
LD12.1	Turbot *S. maximus*	Spain	2001
RM256.1	Turbot *S. maximus*	Spain	2002
LPV1.7	Turbot *S. maximus*	Spain	1995
RI93.1	Turbot *S. maximus*	Spain	2002
ACR104.1	Turbot *S. maximus*	Spain	2001
RM268.1	Turbot *S. maximus*	Spain	2002
RM261.1	Turbot *S. maximus*	Spain	2002
PC538.1	Gilthead sea bream *Sparus aurata*	Spain	2002
PC560.1	Gilthead sea bream *S. aurata*	Spain	2002
DOB102	Gilthead sea bream *S. aurata*	Spain	2002
342101	Gilthead sea bream *S. aurata*	Spain	2001
NCIMB 2158	Sole *S. solea*	UK	1981
NCIMB 2153 ATCC43397	Black sea bream *Acanthopagrus schlegeli*	Japan	1976
NCIMB 2154^T ATCC 43398	Red sea bream *Pagrus major*	Japan	1977

according to the methods described by Sørensen & Larsen (1986). In some assays, absorbed antisera with the heterologous antigens were employed following the procedures of Romalde et al. (1993).

The slide agglutination tests were conducted as previously described by Toranzo et al. (1987), by mixing a drop of each O-antigen suspension with a similar volume of the 1:10 diluted antiserum raised against sole and turbot isolates, respectively, on a multi-well glass slide. Strong and rapid agglutination was registered as a positive result.

Quantitative agglutination tests were performed in 96-well round-bottomed microtiter plates (Corning, New York) using the suspensions of O-antigens from the homologous and heterologous strains (Stevenson & Daly 1982). The agglutination titer was considered the reciprocal of the highest dilution of the antiserum giving a positive reaction after overnight incubation with the antigen at 15°C.

Dot-blot analysis was performed as described by Cipriano et al. (1985). Briefly, O-antigens obtained from each strain were dotted onto nitrocellulose membranes (0.45 µm HA filter, Millipore) and were blocked for 1 h with 3% gelatin (Oxoid) in Tris-buffered saline, TBS (pH 7.5). After washing twice with TBS supplemented with 0.05% Tween 20 (TBS-T), the membranes were exposed to the unabsorbed and absorbed antisera diluted 1:1000 in TBS containing 1% gelatin (TBS-1) for 60 min. The filters were then washed twice with TBS-T and incubated with goat anti-rabbit immunoglobulin G (diluted at 1:3000), with alkaline phosphatase as the conjugate (Bio-Rad). The immunoreactive point was visualized using 0.1 M carbonate buffer (pH 9.8) containing 4-nitro blue tetrazolium chloride (0.3 mg ml^{-1}, Oxoid) and 5-bromo-4-chloro-3-indolylphosphate (0.15 mg ml^{-1}, Oxoid). Only a reaction similar to that exhibited by the homologous strain was scored as positive.

Extraction of lipopolysaccharides (LPS) and electrophoretic analysis. The extraction of LPS present in the cell envelope of the isolates was performed using the methods of Hitchcock & Brown (1983). Bacteria were grown on FMM agar for 72 h, suspended in PBS, and double-washed by centrifugation. The cellular pellet was resuspended in PBS at a concentration of 10^9 cells ml^{-1}. Portions (1.5 ml) of these suspensions were centrifuged at 10 000 × *g* for 2 min to obtain the pellet, which was then suspended in 200 µl PBS and boiled at 100°C for 10 min. The samples were then ice-cooled and treated with proteinase-K solution (2.5 mg ml^{-1}) in PBS, and the mixture was incubated at 60°C for 1 h.

Samples (15 µl) were run submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970), with 12 % (w/v) acrylamide in the resolving gel and 4 % (w/v) acrylamide in the stacking gel, using a Mini Protean® 3 Cell apparatus (Bio-Rad).

Analysis of membrane proteins. The cell envelope proteins from *Tenacibaculum maritimum* strains were prepared from bacterial culture grown on FMM broth following the procedures of Crosa & Hodges (1981). The cultures were centrifuged at 10 000 × *g* for 10 min at 4°C, the pellets were resuspended in 3 ml of 10 mM Tris-HCl (pH 8.0) containing 0.3 % NaCl, and sonicated 3 times with a Bronson sonifier 250 apparatus (60 W, 30 s). After centrifugation for 1–2 min to eliminate cell debris, the resultant supernatants were centrifuged again at 17 000 × *g* for 60 min. The outer membrane fraction was obtained by the method of Filip et al. (1973) with 3 % (w/v) sodium lauryl sarcosinate (Sigma) in 20 mM Tris-HCl (pH 8.0) at room temperature for 20 min to dissolve the inner membrane. Outer membrane material was collected by centrifugation at 17 000 × *g* for 1 h and washed twice with distilled water. The resultant pellets of both methods were kept at –30°C until use.

Total and outer membrane protein were examined by SDS-PAGE, using the same acrylamide concentrations as for LPS, and the standard protein markers ('wide range color markers') were obtained from Sigma. After electrophoresis (60 V for 90 min), proteins were stained with 0.05 % Coomassie blue R (Sigma) in 25 % propan-2-ol-10 % acetic acid. Gels were distained with 10 % acetic acid, 40 % methanol and photographed.

Immunoblotting analysis of protein and LPS. According to the procedures of Towbin et al. (1979), LPS and total protein membranes separated by SDS-PAGE were transferred onto nitrocellulose membrane with a transfer buffer composed of 25 mM TrisHCl (pH 8.3), 192 mM glycine and 20 % methanol, by electroblotting at 350 mA for 1 h in a semi-dry transblot apparatus (Mini Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad). After transfer, immobilized LPS and proteins were detected using unabsorbed and absorbed antisera raised against *Tenacibaculum maritimum* strains from sole and turbot, as outlined above for the dot-blot assay.

RESULTS AND DISCUSSION

Biochemical characterization

The PCR analysis identified the 29 isolates studied as *Tenacibaculum maritimum* strains, with a single 1080 bp band identical to the reference strains (data not shown). Biochemical homogeneity was seen among the *T. maritimum* reference strains and all other strains regardless of the source of isolation. The phenotypic tests showed that all bacterial isolates were Gram-negative, long, slender rods with gliding motility, that produced catalase and cytochrome oxidase. Colonies were flat, pale yellow, with an irregular margin, and adhered strongly to the FMM agar. All isolates absorbed Congo red, but did not contain a cell-wall-associated flexirubin-type pigment. Although Bernardet et al. (1990) and Ostland et al. (1999) have reported that *T. maritimum* typically reduces nitrate and does not produce hydrogen sulfide, in our study, 2 isolates (PC460.1 and LPV 1.7) from turbot, as well as another strain from sole (PC529.1) failed to reduce nitrate and to produce hydrogen sulfide, respectively. However, these atypical results were also found by Chen et al. (1995) for some *Flexibacter maritimus* strains from wild American fish species.

The presence and activity of 19 enzymes in the API ZYM gallery showed that all isolates were similar in number of detected enzymes and level of enzymatic activity produced, i.e. the typical profile of the *Tenacibaculum maritimum* reference strains. The results of our API ZYM tests agree with the findings of other studies (Bernardet et al. 1990, Pazos et al. 1993, Chen et al. 1995). In addition, all *T. maritimum* strains presented an identical sensitivity pattern, being totally resistant to oxolinic acid and highly sensitive to the other chemotherapeutics tested in this study.

Serological characterization

Slide agglutination assays using 2 antisera and O-antigens revealed cross-reactions for all strains regardless of host origin and serum employed. The results of microtiter agglutination tests using unabsorbed sera ranged from 16 to 256 (Table 2). Besides the fact that both antisera presented relatively low titers, even with the homologous strains, their range was similar to that reported by Wakabayashi et al. (1984), who also observed a cross-reaction among different *Tenacibaculum maritimum* strains isolated from red and black sea bream.

However, the dot-blot assays revealed antigenic heterogeneity, with 3 main patterns of serological reactions evident (Table 2). The *Tenacibaculum mari-*

Table 2. *Tenacibaculum maritimum.* Results of microagglutination test, dot-blot assay and Western blot analysis with O-antigens and unabsorbed and absorbed rabbit antisera raised against Isolates PC503.1 from sole and PC424.1 from turbot. ++: strong and immediate reaction; +: delayed positive reaction; –: negative reaction, a reaction was considered positive if it was similar to the reaction exhibited by the strain used to obtain the rabbit sera; *: different result detected with absorbed and unabsorbed antisera. Antigens used to absorb the serum are shown in parentheses. LPS: lipopolysaccharides

Antigen	Microtiter agglutination with serum anti-		Dot-blot assay with serum anti-				Western LPS analysis with serum anti-			
	PC503.1	PC424.1	PC503.1	PC424.1	PC503.1 (PC424.1)	PC424.1 (PC503.1)	PC503.1	PC424.1	PC503.1 (PC424.1)	PC424.1 (PC503.1)
Serotype O1										
Sole (11 strains)	128–256	16–32	++	+	++	–*	++	–	++	–
Gilthead sea bream (4 strains)	64–128	16–32	++	–	++	–	++	–	++	–
Serotype O2										
Turbot (11 strains)	16–32	64–128	–	++	–	++	–	++	–	++
Sole (1 strain)	32	32	–	++	–	++	–	++	–	++
NCIMB 2158	32	32	–	++	–	++	–	++	–	++
Serotype O1/O2										
LR2P	128	32	++	+	++	–*	++	++	++	–*
AZ202.1	256	32	++	+	++	–*	++	++	++	–*
NCIMB 2153	32	32	+	+	+	–*	++	++	++	–*
NCIMB 2154^T	32	32	+	+	–*	+	++	++	–*	+

timum strains isolated from gilthead sea bream reacted only with the antiserum obtained against the sole isolate, the turbot isolates only showed reactivity with the serum raised against the turbot strain, and finally, the majority of sole isolates showed a strong reaction with the antiserum against the sole strain and also cross-reactions with the serum against the turbot strain. These cross-reactions disappeared when the turbot antiserum was absorbed with a heterologous sole strain. Thus, in accordance with the results obtained with absorbed antisera, the isolates were provisionally divided into 2 serological groups. Group 1 comprised most of the strains isolated from sole and the isolates from gilthead sea bream, and Group 2 corresponded to turbot isolates. The reference strains from Dover sole (NCIMB 2158) and red sea bream (NCIMB 2154^T) displayed a similar pattern to Group 2, while the reference strain from black sea bream (NCIMB 2153) appeared to belong to Group 1. These results are in agreement with our preliminary findings using a lower number of strains (Avendaño et al. 2003).

Immunoblot analysis of the LPS clearly revealed 2 major serological groups, that were distinguishable without the use of absorbed antiserum: Serotypes O1 and O2 (Fig. 1). Serotype O1 was comprised of the majority of the sole strains and all gilthead sea bream isolates, which showed a strong immunological reaction with the serum raised against the sole isolate; no reaction was observed among these strains and the serum raised against the turbot isolate. Serotype O2 consisted of all turbot strains and sole strains IEO 8.1 and NCIMB 2158, strains which all showed a strong reaction with the serum raised against the turbot isolate but no reaction with the serum raised against the sole isolate. In general, the immunoblot assays revealed that each serotype presented a characteristic LPS pattern reflected by the inter-band distances as well the position of the core. Probably, proteolytic digestion after proteinase-K treatment eliminates the cross-reactions observed in the dot-blot assays with unabsorbed turbot antiserum.

An intermediate minor serotype, O1/O2, was identifed for 2 sole strains (LR2P and AZ202.1) and the Japanese reference strains NCIMB 2153 and 2154^T, whose LPS displayed a strong antigenic reaction with both unabsorbed antisera (Fig. 1). However, these cross-reactions disappeared in all strains when the absorbed sera were employed. In fact, whereas Reference Strain NCIMB 2154^T showed a pattern similar to that of Serotype O2, the serotypes of the other 3 strains appeared to be more similar to that of Serotype O1.

To date, serological studies on *Tenacibaculum maritimum* have been rare. Although Wakabayashi et al. (1984) claimed antigenic homogeneity of this species based on slide agglutination assays, further studies of Ostland et al. (1999) employing immunoblot analysis of LPS demonstrated antigenic differences among *T. maritimum* isolates from Atlantic salmon *Salmo salar*, suggesting that this microorganism may not be as homogeneous as previously thought. Pazos (1997) reported that different serological subgroups among *T. maritimum* isolates could only be detected when

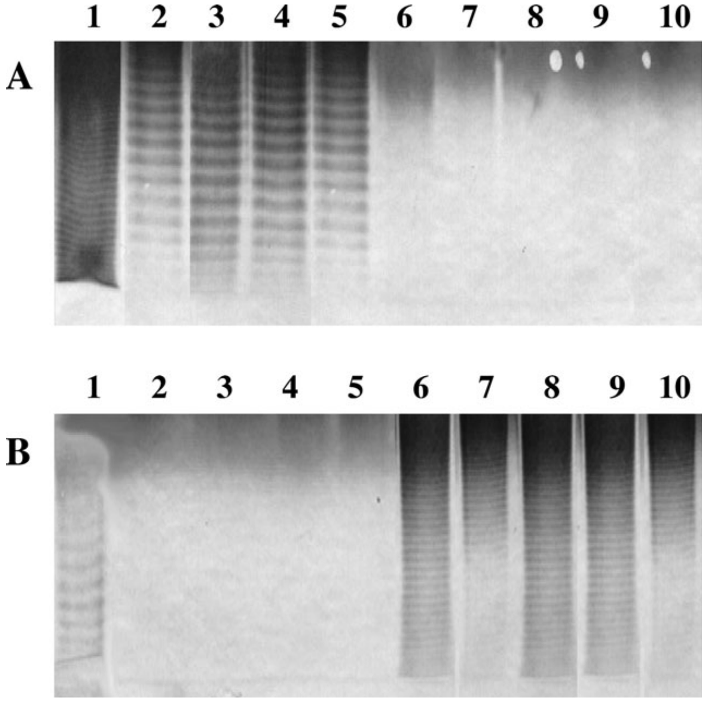

Fig. 1. *Tenacibaculum maritimum.* Western blot analysis of lipopolysaccharides of strains, using antisera raised against (A) sole strain PC503.1 and (B) turbot strain PC424.1 Lane 1: NCIMB 2153; 2: PC477.1; 3: PC503.1; 4: AZ203.1; 5: PC560.1; 6: PC424.1; 7: PC394.1; 8: RI93.1; 9: RM256.1; 10: LPV1.7. Lane 1: Serotype O1/O2 strain; Lanes 2 to 5: Serotype O1 isolates; Lanes 6 to 10: Serotype O2 isolates

absorbed antisera are used in the immunological analysis. However, our findings using dot-blot assays and immunoblot analysis demonstrated the existence of antigenic heterogeneity without the use of absorbed antisera. In addition, the LPS analysis revealed a precise differentiation that has been determinant in the identification of 2 major serotypes, demonstrating definitely that the main differences between the *T. maritimum* strains are caused by O-antigens.

In the case of Serotype O1/O2, it is important to note that its electrophoretic pattern suggests the possibility that the strains of this group behave as chimeras. Similar results have been found for *Flexibacter psychrophilum* in which the existence of 3 major serotypes and several intermediate minor serotypes was reported (Madsen & Dalsgaard 2000, Madetoja et al. 2002).

The analysis of total and outer membrane proteins revealed similar banding patterns among the different serogroups of *Tenacibaculum maritimum*. In fact, all strains shared a considerable number of common bands between 14.2 and 66 kDa (Fig. 2). Similar results have been reported by Pazos (1997), who noted that all *T. maritimum* isolates studied contained a large number of apparently very similar proteins, with molecular masses between 14.4 and 97.4 kDa. These results agree with the studies of Bernardet et al. (1994), who defined *T. maritimum* as a homogenous species by reason of the composition of its proteic cell envelope. These proteins are antigenically related, since a strong reaction was observed with both sera in the Western blot analysis (data not shown).

In conclusion, the results of this study suggest that *Tenacibaculum maritimum* strains are biochemically homogeneous and antigenically different, mainly due to differences in the ‘O’ chains of LPS. Antigenic heterogeneity was established using unabsorbed sera, revealing the existence of 2 major O-serotypes in *T. maritimum* from strictly marine fishes which could be host-specific. However, the serological scheme proposed here could certainly be extended if further studies including more strains of *T. maritimum* isolated from different hosts and/or geographical origins are made.

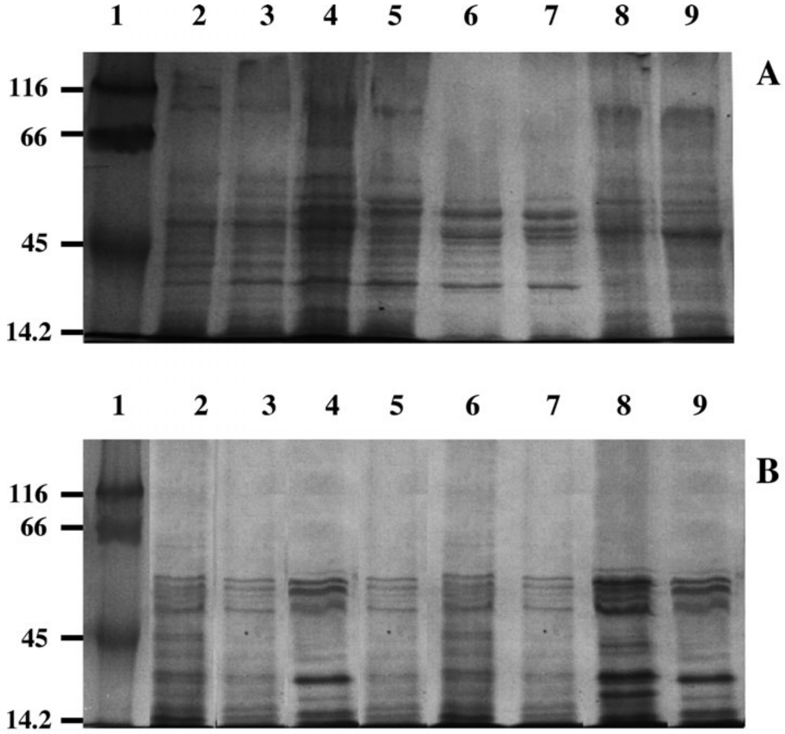

Fig. 2. SDS-PAGE of (A) total and (B) outer membrane proteins of *Tenacibaculum maritimum* strains. Lane: 1: molecular size markers; 2: PC424.1; 3: RM256.1; 4: NCIMB 2153; 5: NCIMB 2158; 6: LR2P; 7: PC528.1; 8: PC503.1; 9: PC560.1. Numbers on left: positions of molecular size marker (kDa)

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A novel O-serotype in *Tenacibaculum maritimum* strains isolated from cultured sole (*Solea senegalensis*)

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Abstract

Tenacibaculum maritimum was consistently isolated from tenacibaculosis outbreaks affecting sole farms in Portugal and south of Spain in the last two years. These new *T. maritimum* isolates could not be assigned within the two major serotypes (O1 and O2) already described. Rabbit antiserum was prepared against one Portuguese sole isolate to examine the antigenic relationships between the isolates from sole using microtitre agglutination tests, dot blot assay and immunoblotting of lipopolysaccharides. Serological characterization of the recent sole isolates demonstrated that they belong to a novel O-serotype named O3, allowing us to extend the serological scheme for this fish pathogen. This information is useful for epizootiological and vaccination studies.

Tenacibaculosis or flexibacteriosis is recognized as an important infectious disease in marine fish since 1979 (Hikida et al., 1979; McVicar and White, 1979). The presence of the etiological agent *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) was first demonstrated by Bernardet et al. (1990) and since then, this pathogen has spread among a wide variety of host species, producing significant losses in cultured fish such as turbot (*Scophthalmus maximus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus kisutch*), sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*) (Devesa et al., 1989; Pazos et al., 1993; Bernardet et al., 1994; Ostland et al., 1999) and, lately, in sole (*Solea solea* and *S. senegalensis*) (Cepeda and Santos, 2002; Avendaño-Herrera et al., 2004).

In previous work we analysed the antigenic diversity of *T. maritimum* from sole, gilthead sea bream and turbot, which allowed us to propose a O-serotyping scheme for *T. maritimum* composed by two major serotypes (O1 and O2) mainly associated with the host species (Avendaño-Herrera et al., 2004). However, since the middle of 2003, new outbreaks of tenacibaculosis have occurred in sole farmed in Portugal and south of Spain. These new *T. maritimum* isolates could not be assigned within the serotypes already described. We report here the existence of a

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new serotype within this fish pathogen, which was proposed as serotype O3.

Twelve *T. maritimum* strains isolated from sole in Portugal and south of Spain were used in the present study. The bacterial strains were confirmed as *T. maritimum* using the PCR-based analysis described by Toyama et al. (1996). The bacteria were routinely cultivated on *F. maritimus* Medium (FMM) (Pazos et al., 1996) at 24°C for 72 h. Stock cultures were maintained frozen at –70°C in Criobille tubes (AES Lab., France).

The antigenic analysis were carried out using the thermostable antigens of each strain obtained after heat killing the bacterial suspensions (10^9 cells ml^{-1}) in phosphate buffered saline (pH 7.4) at 100°C for 60 min, washed once in the same saline solution and maintained at 4°C until required. The Portuguese sole strain ACC13.1 was selected and used to obtain immune serum in rabbit according to the methods described by Sørensen and Larsen (1986). The serological analysis were performed with the unabsorbed and absorbed rabbit serum. For the absorption, the serum against isolate ACC13.1, as well as antisera against strains PC503.1 from sole (serotype O1) and PC424.1 from turbot (serotype O2) (Avendaño-Herrera et al., 2004), were mixed with the antigens of the heterologous isolates and incubated overnight at 4°C. This process was repeated twice to ensure a complete absorption of common antigens.

The agglutination titers of the antiserum ACC13.1 were measured against the suspensions of O-antigens from the homologous and heterologous strains (Stevenson and Daly, 1982). The serological characterization of the twelve *T. maritimum* isolates was performed employing the dot blot assays as described by Cipriano et al. (1985) with the unabsorbed and absorbed antiserum

	Microtitre agglutination			**Dot blot assay**			
	With serum anti-			**With serum anti-**			
Antigen	**PC503.1 (O1)** [a]	**PC424.1 (O2)**	**ACC13.1 (O3)**	**PC503.1 (O1)**	**PC424.1 (O2)**	**ACC13.1 (O3)**	**Absorbed** [b]
Serotype O1							
Sole strain PC503.1	256	16	64	++	+	-	- *
Serotype O2							
Turbot strain PC424.1	16	128	32	-	++	-	++
Serotype O3							
12 sole isolates	32	32	256	-	+	++	- *

[a] Parenthesis indicate the serotype.

[b] Serum anti-PC424.1 absorbed with the heterologous sole strain ACC13.1.

++, strong and immediate reaction; +, delayed positive reaction; -, negative reaction. Asterisks indicate different result detected with absorbed and unabsorbed antisera.

Table 1. Results of microagglutination test and dot blot assay with O-antigens and rabbit antisera raised against the *T. maritimum* isolates.

obtained against the ACC13.1 isolate and each representative of serotypes O1 and O2.

In order to evaluate the antigenic variability among strains, we characterized the isolates analyzing their lipopolysaccharides (LPS) following the procedures of Hitchcock and Brown (1983). Samples were examined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE; Laemmli, 1970) using 12% acrylamide in the resolving gel and 4% acrylamide in the stacking gel. LPS components separated by SDS-PAGE were transferred onto nitrocellulose membrane by electrophoretic blotting according to procedures of Towbin et al. (1979), and treated for immunological analysis as outlined above for the dot blot assay.

The results of microtitre agglutination tests using unabsorbed serum showed the highest titres with the homologous strains, providing evidence of antigenic specificity (Table 1). When the dot blot assays were performed, the present sole isolates studied showed strong reaction with the antiserum raised against the Portuguese sole strain (Figure 1a). Although weak cross-reactions with the serum against the serotype O2 were observed, they fully disappeared when the O2 antiserum was absorbed with the heat stable O-antigen of the heterologous Portuguese sole strain, confirming that all the new sole isolates correspond to a distinct *T. maritimum* serological group (Table 1).

The immunoblot of the LPS clearly assured the existence of this novel serological group within *T. maritimum*, which was distinguished without the necessity to use absorbed antiserum in agreement with our previous findings (Avendaño-Herrera et al., 2004). This serotype named O3 comprised all the sole strains previously untypable, which showed a strong immunological reaction of their O side chains when the unabsorbed serum raised against the sole isolate ACC13.1 was used (Figure 1b). As expected no reaction with the antisera raised against the representative of serotypes O1 and O2 was detected (Figure 1b).

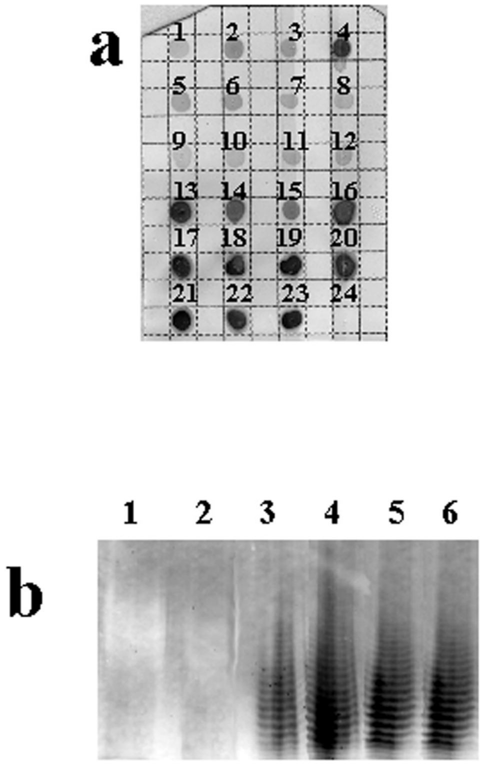

Figure 1. Dot blot assay (a) and immunoblot of the LPS (b) of the isolates of *T. maritimum*, using the antiserum raised against the Portuguese sole isolate ACC13.1. In 1a, strain ACC13.1 (dot 4) and saline solution (0.85% wt/vol NaCl)(dot 24) were used as positive and negative controls respectively. Dots: 1 and 5 to 8, serotype O1 strains; 2 and 9 to 12, serotype O2 strains; 3, serotype O1/O2 strain; 13 to 23, new serotype O3 isolates. Lanes in 1b: 1, serotype O1 strain (PC503.1); 2, serotype O2 strain (PC424.1); 3 to 6, new serotype O3 isolates.

Until now the majority of *T. maritimum* isolated from sole in northwestern of Spain belonged to serotype O1 (Avendaño-Herrera et al., 2004). This antigenic heterogeneity would warrant further investigation because serologically distinct *T. maritimum* strains may be associated with different outbreaks. Moreover, often sole farms in the Iberian peninsula do not posses the whole fish cycle, importing or moving fish from other areas, which suggest the possible coexistence of *T. maritimum* serotypes O1 and O3 in a same farm. Further studies are necessary to elucidate this question. Therefore, our finding here must be taken into account in order to consider the incorporation of the new serotype O3 antigens in the vaccine formulation against tenacibaculosis in sole culture.

Acknowledgement

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III.B. LÁMINAS COMPLEMENTARIAS A LAS PUBLICACIONES

Lámina 1. Morfología de las células de *Tenacibaculum maritimum* mediante tinción con cristal violeta (A). Las células tienen una forma de bacilo alargado con un tamaño de 0,5 µm x 2-30 µm. Morfología celular observada en un frotis directo de la zona de la boca de un pez con problemas de tenacibaculosis (B).

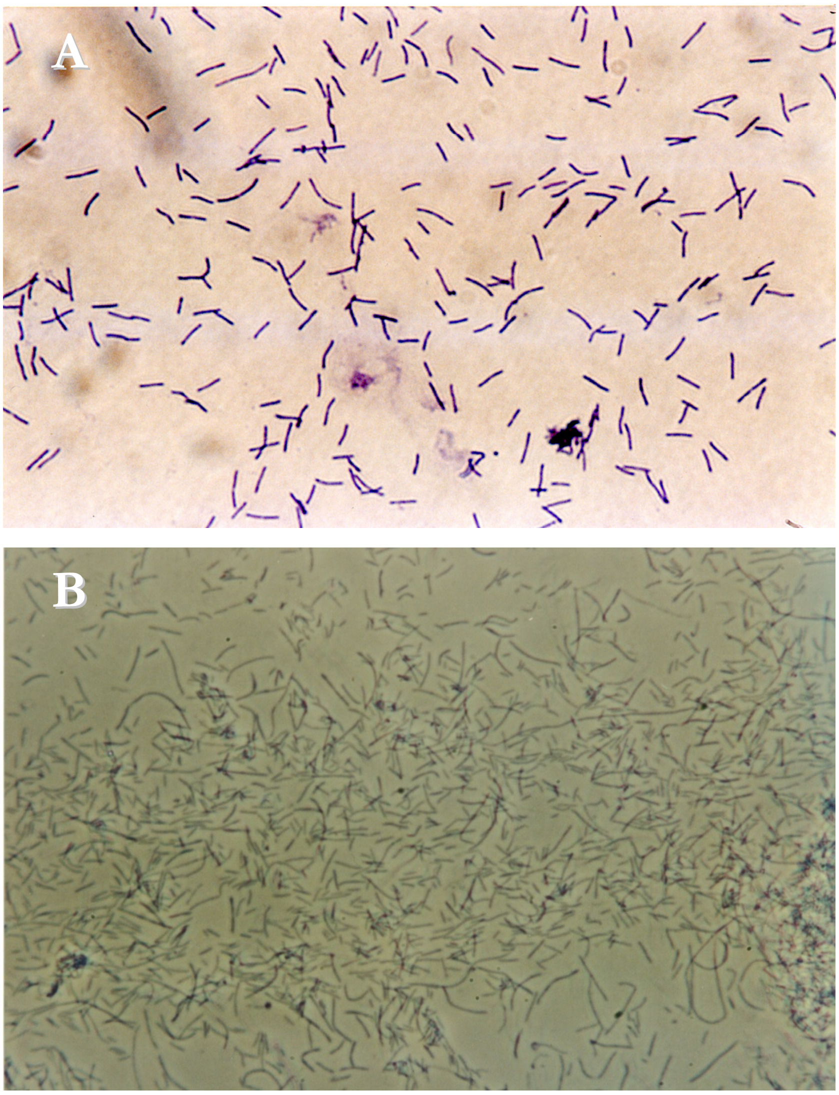
Lámina 2. Perfil bioquímico obtenido en el sistema convencional API ZYM con las células de *Tenacibaculum maritimum*.

Lámina 3. Resultados de algunas pruebas enzimáticas de degradación con cepas de *Tenacibaculum maritimum* como gelatina (A); lecitina (B); amilasa (C) y quitina (D).

Lámina 4. Análisis de LPS mediante la técnica de "immunoblot" empleando el antisuero obtenido a partir de (A) la cepa de lenguado PC503.1 y (B) cepa de rodaballo PC424.1. Líneas: 1: NCIMB 2153; 2: PC477.1; 3: PC503.1; 4: AZ203.1; 5: PC560.1; 6: PC424.1; 7: PC394.1; 8: RI93.1; 9: RM256.1; 10: LPV1.7. Líneas 1: Cepa Serotipo O1/O2; Líneas 2 a 5: Aislados Serotipo O1; y Líneas 6 a 10: Aislados Serotipo O2.

Lámina 5. Análisis de las proteínas de membrana (A) totales y (B) externas de cepas de *Tenacibaculum maritimum* en geles de SDS-PAGE. Línea 1: marcador de peso molecular; 2: PC424.1; 3: RM256.1; 4: NCIMB 2153; 5: NCIMB 2158; 6: LR2P; 7: PC528.1; 8: PC503.1; 9: PC560.1. Los números de la izquierda indican la masa molecular del marcador, expresada en kilodaltons (kDa).

Lámina 1.



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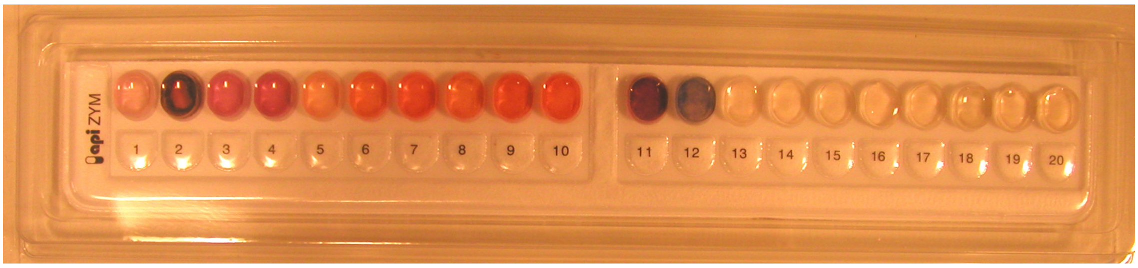

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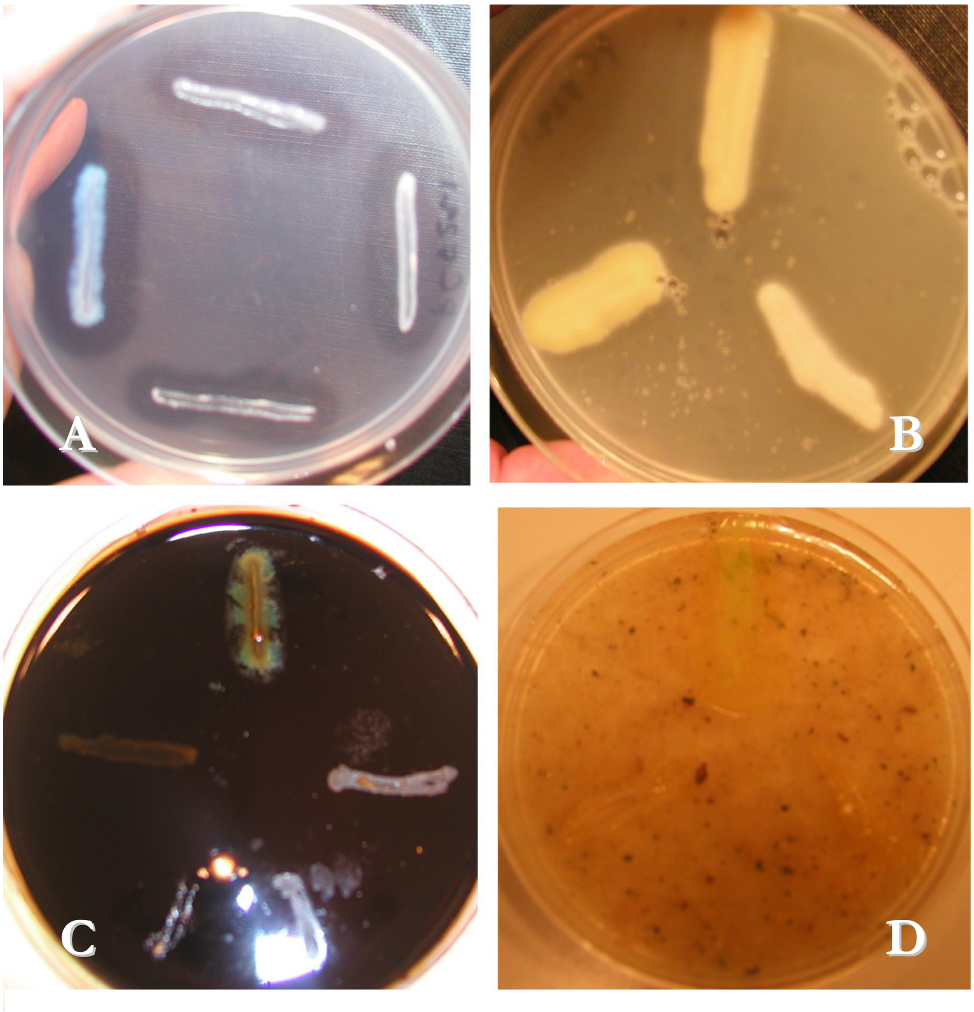

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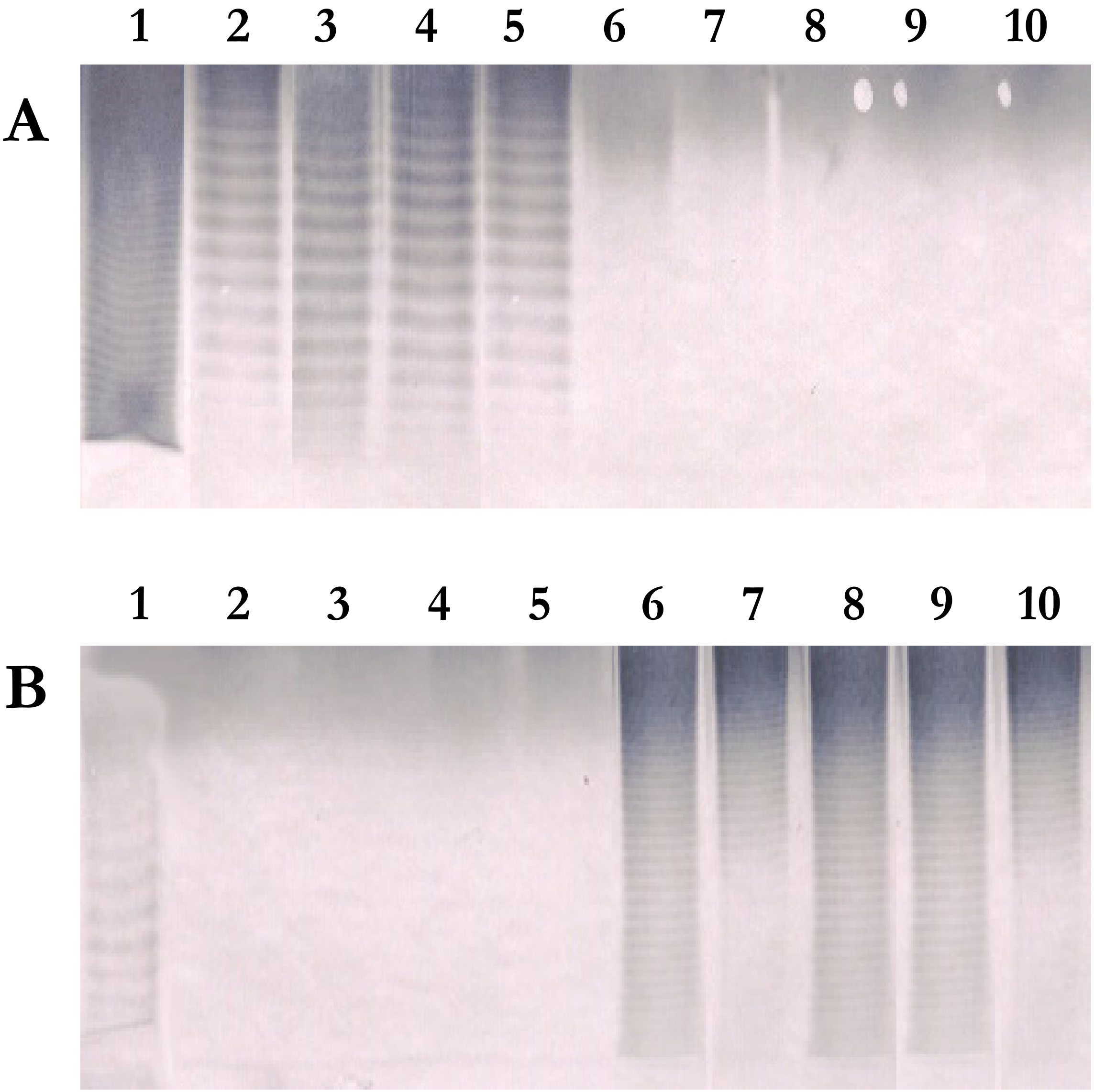

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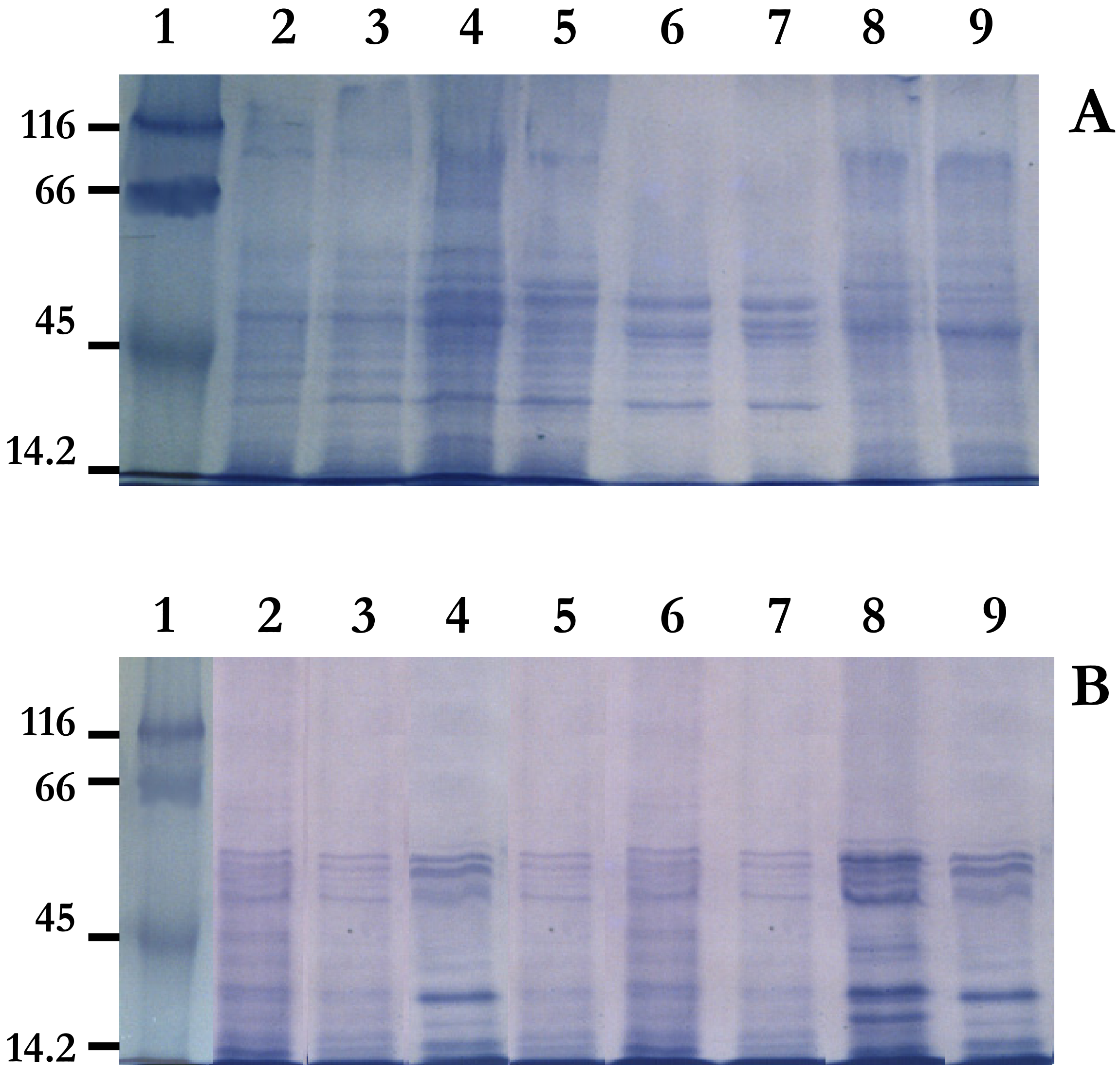

IV. CARACTERIZACIÓN MOLECULAR DE LAS CEPAS DE *Tenacibaculum maritimum*

IV.A. PUBLICACIONES

Artículo nº 3: Intraspecific diversity of the marine fish pathogen *Tenacibaculum maritimum* as determined by randomly amplified polymorphic DNA-PCR. **Journal of Applied Microbiology (2004). 96**: 871-877.

CONTENIDO:

- Extracción del material cromosómico.
- Estudio del genoma mediante la técnica de la amplificación aleatoria del ADN.

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Intraspecific diversity of the marine fish pathogen *Tenacibaculum maritimum* as determined by randomly amplified polymorphic DNA-PCR

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ABSTRACT

R. AVENDAÑO-HERRERA, J. RODRÍGUEZ, B. MAGARIÑOS, J.L. ROMALDE AND A.E. TORANZO. 2004.

Aim: The aim of the present study was to evaluate the intraspecific genetic variability within *Tenacibaculum maritimum* strains isolated from different species of marine fish.

Methods and Results: Twenty-nine strains isolated from five different fish species and three reference strains were characterized by randomly amplified polymorphic DNA (RAPD) method. Cluster analysis of RAPD-PCR profiles showed that the strains, regardless of the oligonucleotide primer employed (P2 and P6), were separated into two main groups that strongly correlated with the host species and/or O-serotypes described for this pathogen. One group composed all strains isolated from sole (*Solea senegalensis* and *S. solea*) and gilthead seabream (*Sparus aurata*), and the other compiled the *T. maritimum* isolates from yellowtail (*Seriola quinqueradiata*), Atlantic salmon (*Salmo salar*) and turbot (*Scophthalmus maximus*). An important exception was observed in the RAPD patterns of the reference strains, which were included in different genetic groups depending on the primer employed.

Conclusions: The results obtained demonstrated genetic variability within the *T. maritimum* isolated from different marine fish. Such genetic variability proved to be strongly associated with the host and/or serogroups described for this pathogen.

Significance and Impact of the Study: The RAPD analysis constitutes a valuable molecular technique for epidemiological studies of *T. maritimum*. Interestingly, this is the first report of intraspecific differentiation and characterization of *T. maritimum* strains isolated from cultured fish.

Keywords: intraspecific diversity, marine fish, RAPD analysis, *Tenacibaculum maritimum*.

INTRODUCTION

Tenacibaculum maritimum (formerly *Flexibacter maritimus*) is the causal agent of flexibacteriosis in marine fish since 1979 (Hikida *et al.* 1979; McVicar and White 1979). Since 1990, as the farming of fish became a steadily growing industry, this disease has been widely distributed in Japan, North America, several countries in Europe and Australia, and is considered a limiting factor for the culture of many species of commercial value in distinct geographical areas (Wakabayashi *et al.* 1986; Devesa *et al.* 1989; Bernardet *et al.* 1990, 1994; Pazos *et al.* 1993; Chen *et al.* 1995; Handlinger *et al.* 1997; Ostland *et al.* 1999). In addition, *T. maritimum* is suspected to be responsible of some fish mortalities in Chile (Bernardet 1998), but the isolates have not been fully identified and, to our knowledge, no confirmation has been published yet. In Spain, the first disease cases were described in turbot (*Scophthalmus maximus*), salmon (*Salmo salar*) and, lately, in sole (*Solea senegalensis* and *Solea solea*) and gilthead seabream (*Sparus aurata*) (Devesa *et al.* 1989; Alsina and Blanch 1993; Pazos *et al.* 1993; Cepeda and Santos 2002; Avendaño-Herrera *et al.* 2004).

Although this bacterium is biochemically homogeneous (Bernardet and Grimont 1989; Bernardet *et al.* 1990; Avendaño-Herrera *et al.* 2004) recent antigenic studies

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revealed that at least two major 'O' serogroups can be detected within *T. maritimum* strains, which seem to be related to host species (Avendaño-Herrera *et al.* 2004). This serological analysis demonstrated antigenic heterogeneity as a good epidemiological marker in this fish pathogen. However, until now, no discriminative methods based on genetic techniques have been applied to this bacterium.

Development of new molecular techniques has progressed rapidly, and the randomly amplified polymorphic DNA (RAPD) has become an accessible and sensitive method which is based on the use of arbitrary primers to amplify polymorphic segments of DNA (Williams *et al.* 1990). Therefore, the determination of RAPD patterns has been successfully employed for strain discrimination within a number of fish and shellfish bacterial pathogens (Williams *et al.* 1990; Aznar *et al.* 1993; Inglis *et al.* 1996; Chakroun *et al.* 1997; Romalde *et al.* 1999; Grayson *et al.* 2000; Magariños *et al.* 2000; O'hIci *et al.* 2000).

In this work, RAPD method has been employed to analyse the intraspecific genetic variability within *T. maritimum* strains from different species of marine fish, with the aim of evaluating this technique as a useful epidemiological tool for this pathogen.

MATERIALS AND METHODS

Bacterial strains

The 32 *T. maritimum* strains included in this study are listed in Table 1. This collection comprises 29 strains isolated from six different species (two species of sole, turbot, gilthead seabream, Atlantic salmon and yellowtail) and three reference strains (NCIMB 2153, 2154^{T} and 2158) from the National Collection of Marine, Food and Bacteria (Aberdeen, UK). The majority of the isolates, selected from our laboratory collection, were obtained from disease outbreaks occurring in different fish farms in Spain over an 8-year period. All bacterial strains were confirmed as *T. maritimum* using the PCR-based analysis described by Toyama *et al.* (1996). For all experiments, the strains were routinely grown on *Flexibacter maritimus* medium (FMM) agar (Pazos *et al.* 1996) and incubated at 20°C for 72 h. Stock cultures were maintained frozen at −70°C in Criobille tubes (AES Lab., Combourg, France).

DNA extraction

Chromosomal DNA was extracted using InstaGene Matrix (Bio-Rad, Madrid, Spain) as previously described by Romalde *et al.* (1999). Briefly, *T. maritimum* colonies were collected on agar plates, suspended in 1 ml of autoclaved water and centrifuged at 12 000 *g* for 1 min. After the supernatants were removed, the pellets were resuspended in 200 μl of InstaGene Matrix and incubated at 56°C for 30 min. The suspensions were then mixed at high speed for 10 s and boiled in a water bath for 8 min. The lysates were mixed again at high speed and spun at 12 000 *g* for 3 min. The concentration and quality of each DNA sample was examined spectrophotometrically at 260 nm and adjusted to a concentration of 10–20 ng μl^{-1}. All DNAs were maintained at −20°C until they were used for PCR reactions. One microlitre of each DNA solution was used in the respective amplification reaction.

RAPD analysis

The random amplified polymorphic DNA (RAPD) reactions were performed using Ready-To-Go RAPD analysis beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). These commercial beads have been optimized for PCR reactions and contain buffer, nucleotides and *Taq* DNA polymerase. The only reagents which must be added to the reaction are template DNA and primers, also supplied in the kit. Six distinct random 10-mer primers (Amersham Pharmacia Biotech) were included in this study: P1 5′-d[GGTGCGGGAA]-3′, P2 5′-d[GTTTCGCTCC]-3′, P3 5′-d[GTAGACCCGT]-3′, P4 5′-d[AAGAGCCCGT]-3′, P5 5′-d[AACGCGCAAC]-3′ and P6 d[CCCGTCAGCA]-3′. Each RAPD reaction consisted of 1 μl of purified bacterial DNA, 1·5units of *Taq* polymerase, 10 mM Tris–HCl (pH 9·0), 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM each dNTP 25 pmol of respective primer in a 25 μl total volume. The amplification protocol for RAPD analyses started with denaturation (95°C for 300 s) followed by 30 cycles at 95°C for 60 s (denaturation), 35°C for 60 s (annealing) and 72°C for 120 s (DNA chain extension) in a T Gradient thermocycler (Biometra, Goettingen, Germany). RAPD products were separated by using horizontal electrophoresis on a 1·5% agarose gel for 90 min at 100 V, stained with ethidium bromide (Bio-Rad) and photographed under ultraviolet light. A 50–2000-pb ladder (Sigma, St Louis, MO, USA) was used as a molecular mass marker. In order to determine significant differences in the patterns, reproducibility of results were assessed by repetition in at least three independent RAPD assays, in which DNAs obtained in two different extractions were employed.

Computer data analysis

All the gels were also scanned and the images were captured by a Gel Doc-2000 gel documentation system (Bio-Rad). For the analysis and comparison of the RAPD patterns, a data analysis was performed by using the Diversity Database software (Bio-Rad). The computed similarities among isolates were estimated by means of the Dice coefficient (S_d) (Dice 1945) as a measure of homology, according to the

Table 1 *Tenacibaculum maritimum* strains used in this study and their distribution in the different RAPD profiles

Bacterial isolate	Host species	Origin	Serotype*	RAPD type	
				P2	P6
PC538.1	*Sparus aurata*	Spain	O1	Ia	1
PC560.1	*Sparus aurata*	Spain	O1	Ia	1
PC824.1	*Sparus aurata*	Spain	O1	Ia	1
PC477.1	*Solea senegalensis*	Spain	O1	Ia	1
PC487.1	*Solea senegalensis*	Spain	O1	Ia	1
PC492.1	*Solea senegalensis*	Spain	O1	Ia	1
PC503.1	*Solea senegalensis*	Spain	O1	Ia	1
PC504.1	*Solea senegalensis*	Spain	O1	Ia	1
PC528.1	*Solea senegalensis*	Spain	O1	Ia	1
PC529.1	*Solea senegalensis*	Spain	O1	Ia	1
PC517.1	*Solea senegalensis*	Spain	O1	Ia	1
PC432.1	*Solea senegalensis*	Spain	O1	Ia	1
AZ203.1	*Solea senegalensis*	Spain	O1	Ib	1
AZ202.1	*Solea senegalensis*	Spain	O1/O2 (O1)	Ib	1
LR2P	*Solea solea*	Spain	O1/O2 (O1)	Ib	1
PC424.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
PC460.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
PC394.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
LD12.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
RM256.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
RI93.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
ACC8.1	*Scophthalmus maximus*	Portugal	ND	IIa	2a
RM268.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
LD29.1	*Scophthalmus maximus*	Spain	O2	IIa	2a
PC473.1	*Scophthalmus maximus*	Spain	O2	IIa	2b
PC682.1	*Scophthalmus maximus*	Spain	O2	IIb	2a
ACR104.1	*Scophthalmus maximus*	Spain	O2	IIb	2b
SO127	*Salmo salar*	Spain	O2	IIa	2a
DBA4a†	*Seriola quinqueradiata*	Japan	O1/O2 (ND)	IIa	2b
NCIMB 2153	*Acanthopagrus schlegeli*	Japan	O1/O2 (O1)	IIb	2b
NCIMB 2154^T	*Pagrus major*	Japan	O1/O2 (O2)	IIa	3
NCIMB2158	*Solea solea*	UK	O2	IIa	3

RAPD, randomly amplified polymorphic DNA.

*Serotypes following the typing schema of Avendaño-Herrera *et al.* (2004). The serotype detected with absorbed antisera is shown in parenthesis. ND means serotype not determined.

†Kindly supplied Dr J.F. Bernardet (Unité de Virologie et Inmunologie Moleculaires. INRA, France).

equation: $S_d = [2A/(2A + B + C)] \times 100$, where A is the number of matching band and B and C are the numbers of bands present in one strain but not in the other. Dendrograms were produced on the basis of the unweighted average pair group method (UPGMA).

RESULTS

RAPD analysis

The PCR analysis allowed the confirmation of the 29 isolates studied as *T. maritimum* strains, observing a single product with the expected 1080 bp length identical to the three reference strains (data not shown).

Before the RAPD technique was applied to the 32 isolates of *T. maritimum*, the six distinct random 10-mer primers were screened with purified genomic DNA of two isolates from different hosts (PC503.1 and PC424.1). Only three of the six primers tested (primers P2, P4 and P6) generated an appropriate pattern of amplified products suitable for accurate analysis and were selected for further studies with all strains. The primers P1 and P3 yielded a low number of amplification bands, which were not sufficient for an accurate genetic study of this pathogen, while with the oligonucleotide P5 one isolate gave no amplification pattern (data not shown).

The RAPD analysis with the sequence of the primer P2 allowed us to clearly differentiate two genetic groups (I and

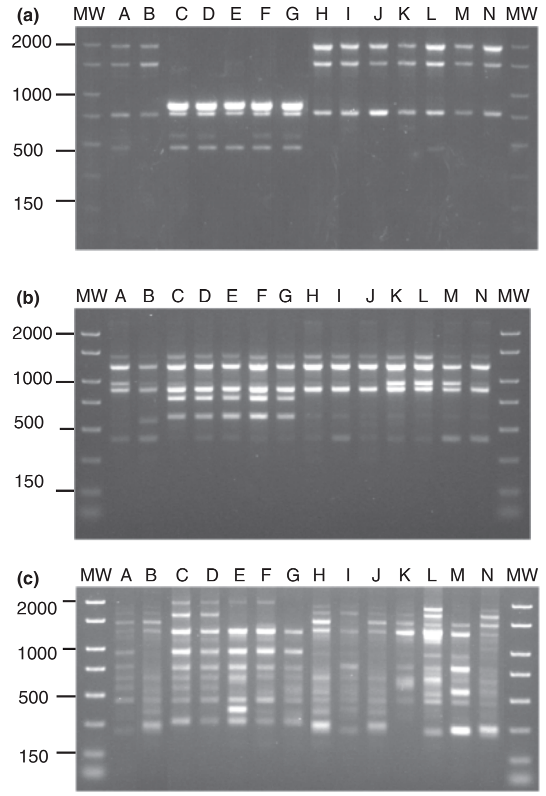

Fig. 1 RAPD fingerprints obtained for the *Tenacibaculum maritimum* isolates using primer 2 (a), primer 6 (b) and primer 4 (c). Lanes: MW, AmpliSize Molecular Ruler (50–2000-pb ladder; Sigma); A – NCIMB 2153; B – NCIMB 2158; C – PC503.1; D – PC529.1; E – AZ203.1; F – PC538.1; G – PC560.1; H – PC424.1; I – LD12.1; J – RI93.1; K – PC473.1; L – ACR104.1; M – DBA4a; N – SO127. Numbers on the left indicate the position of molecular size marker in kb

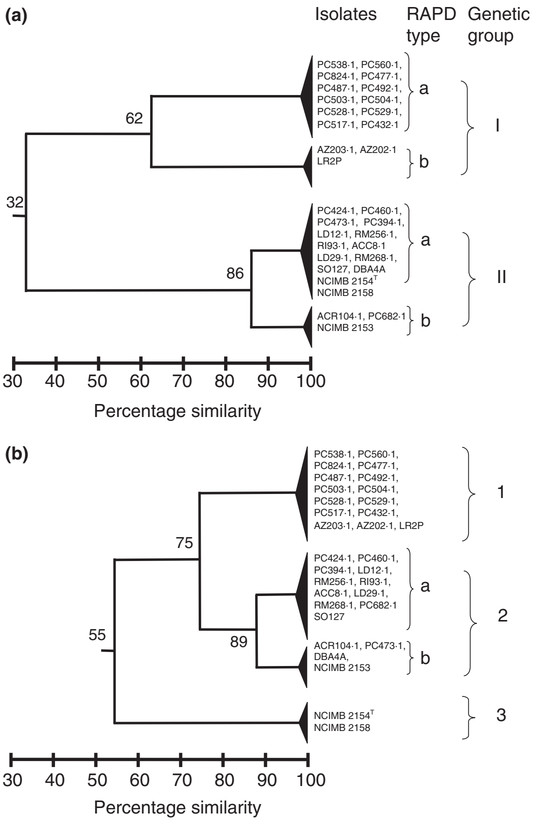

Fig. 2 Dendrograms established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of *Tenacibaculum maritimum* strains obtained with primer 2 (a) and primer 6 (b)

II) within *T. maritimum*, that seem to be related to the host origin of the isolates (Figs 1a and 2a). Only one band of 755 bp was shared by both groups. A similarity of 32% between the two groups was detected when the Dice coefficient was applied. Within the first group (group I), two different RAPD types were observed and designed as Ia and Ib (S_d of 62%). Profile Ia compiled all strains isolated from gilthead seabream and the majority of strains obtained from sole, with exception of three isolates which were grouped as profile Ib. All strains within the genetic group I showed a profile of four major bands ranging from 500 to 1000 bp in size (Fig. 1a). Two subgroups were also detected within the genetic group II (IIa and IIb). The majority of the turbot isolates were grouped in profile IIa, which also included the *T. maritimum* reference strains NCIMB 2154^T and 2158, as well as the yellowtail and Atlantic salmon isolates. However, the *T. maritimum* reference strain NCIMB 2153 and two isolates from turbot (ACR104.1 and PC682.1) yielded a different RAPD profile, and were designed as subgroup IIb. A similarity of 86% was found between profiles IIa and IIb (Fig. 2a).

When primer 6 was employed, the isolates were separated into three genetic groups with a similarity level of 55% (Fig. 2b), which could also be related to the host species. Group 1 contained all isolates from sole and gilthead seabream included in this study. Group 2 included the rest of the isolates except the reference strains of *T. maritimum* NCIMB 2154^T and 2158, which constituted group 3. Two subgroups could be differentiated within group 2 at a similarity level of 89%. One profile comprised the salmon isolate and the majority of turbot strains (2a) and the other subgroup (2b) corresponded to two turbot isolates (PC473.1

and ACR104.1), the yellowtail strain and the reference strain NCIMB 2153. All isolates from subgroups 2a and 2b shared five of the six major bands present in the profile with the exception of a unique 972 bp fragment (Fig. 1b).

In contrast to the other primers, when primer 4 was employed, practically each *T. maritimum* isolate yielded a strain-specific RAPD profile, characterized for the amplification of more than 15 bands (Fig. 1c).

Reproducibility of RAPD results was assessed by the employment of DNA from different extractions and by repetition in at least three independent RAPD assays for each primer tested. Besides some variations in the band intensity, no differences were observed between the profiles obtained for each strain, and all the banding patterns proved highly reproducible.

DISCUSSION

The phenotypic homogeneity in *T. maritimum* has been detailed in many publications, as it is considered an homogeneous taxon on the basis of different biochemical and physiological characteristics (Wakabayashi *et al.* 1984; Baxa *et al.* 1986; Bernardet and Grimont 1989; Bernardet *et al.* 1990; Chen *et al.* 1995). Recent studies using serological methods have demonstrated the existence of distinct serological groups, intimately associated with the host species (Avendaño-Herrera *et al.* 2004). However, to our knowledge, there are no studies on the genetic diversity of *T. maritimum* and, therefore, the possible correlation among putative clonal lineages and serological groups, as already described for other fish pathogens (Toranzo *et al.* 1997; López-Romalde *et al.* 2003), has not been examined. For this reason, in this study the RAPD analysis was applied to obtain genetic fingerprints of *T. maritimum*, and to evaluate its discriminatory power in order to be employed as useful genotyping method for epidemiological purposes.

RAPD uses a single primer in low-stringency polymerase reaction (Welsh and McClelland 1990; Williams *et al.* 1990). Random binding of primers results in different patterns for isolates with no identical DNA, which can then be grouped into different clusters, thus providing the means to carry out epidemiological investigation. This method has also been used in the study of intra- and interspecific relationships in a wide range of micro-organisms including bacteria and yeast (Baleiras *et al.* 1995; Gutacker *et al.* 2003; Ravelo *et al.* 2003).

The results obtained in this work clearly demonstrated the existence of genetic heterogeneity among *T. maritimum*. The appropriate random primers P2 and P6 were first chosen by comparing the RAPD patterns of the 32 *T. maritimum* strains. Based on these primers, the *T. maritimum* strains were separated into two major genetic groups strongly correlated with the host species and O-serotypes previously reported (Avendaño-Herrera *et al.* 2004). One cluster included all strains isolated from sole and gilthead seabream (serotype O1) and the other compiled the *T. maritimum* isolates from yellowtail, Atlantic salmon and all turbot isolates (serotype O2). An important exception was observed in the RAPD patterns with the reference strains, as they were included in different genetic groups depending of the primer employed.

It is interesting to point out that with primer 2, only one band of 755 bp length was shown by all the isolates. This fragment would certainly be a favourable trait for the development of genetic amplification and hybridization assay for diagnostic purpose (Miyata *et al.* 1996; Dalla Valle *et al.* 2002).

Although primer 4 does not allow the establishment of genetic groups because of its high discriminatory power, it can be useful to follow the spreading of bacterial strain responsible for a particular outbreak, due to the fact that practically each *T. maritimum* strain yielded a particular RAPD profile.

Variability and problems with reproducibility in the RAPD profiles have been reported for several bacterial groups (Cunningham 2002). However, in our case no differences were observed among the profiles obtained for each strain in the independent experiments performed, which included separate DNA extractions and/or several RAPD-PCR reactions. However, it is important to outline that, similarly to previous reports (Dalsgaard 1993), we have not detected any plasmid band in our isolates of *T. maritimum* by the modified Kado method (Toranzo *et al.* 1983). Therefore, the distinct RAPD profiles described here were not influenced by the presence of extra-chromosomal DNA.

In summary, in this work we have demonstrated genetic variability within the *T. maritimum* isolated from different marine fish, which was strongly associated with the host and/or serogroups described for this pathogen. In addition, the RAPD analysis constitutes a valuable molecular technique for epidemiological studies of *T. maritimum*.

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V. DESARROLLO DE TÉCNICAS MOLECULARES PARA EL DIAGNÓSTICO DE *Tenacibaculum maritimum*

V.A. PUBLICACIONES

Artículo nº 4: Species-specific polymerase chain reaction primer sets for the diagnosis of *Tenacibaculum maritimum* infection. **Diseases of Aquatic Organisms (2004). 62:**75-83.

Artículo nº 5: A non-destructive method for rapid detection of *Tenacibaculum maritimum* in farmed fish using nested PCR amplification. 24: 280-284. **Bulletin of the European Association of Fish Pathologists (2004). 24**: 280-286.

CONTENIDOS:

❖ Ensayos de identificación del patógeno "*in vitro*"

- Extracción del ADN a partir de cultivo puro, mixto y diversos tejidos de peces infectados con el patógeno.
- Comparación de la especificidad y sensibilidad de los protocolos de PCR descritos para la identificación de *Tenacibaculum maritimum.*
- Confirmación de los resultados mediante ensayos de restricción del producto de amplificación del genoma.
- Desarrollo de una PCR anidada para una rápida identificación del patógeno y estudios de detección en muestras no destructivas.

❖ Ensayos de diagnóstico en estudios de campo.

- Comparación de la eficacia de la PCR anidada con los métodos de diagnóstico tradicionales usando muestras obtenidas en diferentes piscifactorías españolas.
- Validación del mucus como tejido preferido para la identificación del patógeno.
- Detección de *Tenacibaculum maritimum* a partir de peces portadores asintomáticos.

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Species-specific polymerase chain reaction primer sets for the diagnosis of *Tenacibaculum maritimum* infection

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ABSTRACT: In this study the specificity and sensitivity of 2 primer pairs, MAR1–MAR2 and Mar1–Mar2, for the detection of *Tenacibaculum maritimum* were evaluated in parallel using 79 *T. maritimum* strains isolated from different fish species, as well as 53 representatives of related and unrelated bacterial species. Both primer pairs were species-specific for *T. maritimum*, since no amplification products were obtained from chromosomal DNA of the non-*T. maritimum* bacteria tested. However, whereas MAR1–MAR2 identified all the *T. maritimum* strains studied, producing a unique and clear PCR band of the expected 1088 bp length, the Mar1–Mar2 primer pair failed to amplify the 400 bp specific band in 3 sole isolates. To verify if these strains belonged to *T. maritimum* species, 2 endonucleases (*Pvu*I and *Sac*II) were selected as the most adequate enzymes to confirm the specificity of the MAR1–MAR2 amplified fragment. The digestion patterns obtained with both endonucleases supported the assignation of all the strains to *T. maritimum*. The sensitivity of both PCR detection methods was also different, showing a reduction of sensitivity in at least one order of magnitude of the Mar1–Mar2 primer pair in comparison with MAR1–MAR2. When the MAR–MAR2 PCR protocol was applied to different seeded turbot tissues, the detection limit was 10^2 to 10^4 *T. maritimum* cells per reaction. In addition, a nested PCR protocol for detection of this pathogens based on MAR1–MAR2 was developed, which increased the sensitivity by approximately 2 orders of magnitude, ranging from 1 to 250 *T. maritimum* cells per reaction depending on the tissue employed. The tissues that allowed the most easy detection of *T. maritimum* were the skin and mucus. Based on the findings reported here, we propose the nested PCR protocol as the most adequate for an accurate detection of *T. maritimum* in diagnostic pathology as well as in epidemiological studies of gliding bacterial disease of marine fish.

KEY WORDS: Tenacibaculosis · *Tenacibaculum maritimum* · Nested-PCR · Diagnosis

INTRODUCTION

Diseases caused by bacteria are recognized as major problems affecting the marine fish farming through the world (Austin & Austin 1999, Osorio & Toranzo 2002). Although pathogenic species have been described in most of the existent taxonomic groups, only a small number are responsible for important economic losses in extensive culture worldwide. *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (Suzuki et al. 2001) has been included among these microorganisms, being the causative agent of gliding bacterial disease (or tenacibaculosis) in a great variety of valuable marine fish species such as turbot *Scophthalmus maximus*, salmon *Salmo salar* and *Oncorhynchus kisutch*, sole *Solea senegalensis* and *Solea solea*, gilthead seabream *Sparus aurata*, red and black seabream *Pagrus major* and *Acanthopagrus schlegeli*, and floun-

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der *Paralichthys olivaceus* (McVicar & White 1979, Wakabayashi et al. 1984, Devesa et al. 1989, Bernardet et al. 1990, Pazos et al. 1993, Chen et al. 1995, Ostland et al. 1999, Santos et al. 1999, Bader & Starliper 2002, Avendaño-Herrera et al. 2004a).

The disease is preliminarily diagnosed by the clinical signs of the affected fish which usually show eroded and hemorrhagic mouths, ulcerative skin lesions, frayed fins and tail rot. This diagnosis must be supported by the isolation of colonies of this pathogen on appropriate specific media (Pazos et al. 1996), followed by biochemical identification. This traditional culture-based method for the detection of the pathogen requires several days to weeks before results are obtained. In addition, one of the problems in the study of *Tenacibaculum maritimum* is the difficulty of distinguishing it from other phenotypically similar and phylogenetically related species, particularly those of the genera *Flavobacterium* and *Cytophaga* (Suzuki et al. 2001, Bader & Starliper 2002). Another disadvantage in the diagnosis of this infection is the problem of recognizing the colonies of *T. maritimum* among the mixed population that usually overgrow the *T. maritimum* colonies, due to the slow growth characteristic of *T. maritimum*.

All these features make DNA-based methods a very worthwhile approach for diagnosis of tenacibaculosis. Polymerase chain reaction (PCR) has been established as an alternative to traditional detection methods in microbial diagnostics due to the fact that this technique provides a powerful tool for accurate identification of the pathogen from plate cultures, as well as from fish tissues (Cunningham 2002, Osorio & Toranzo 2002). Two PCR primer pairs have been designed for the detection of *Tenacibaculum maritimum* using the 16S ribosomal RNA (rRNA) gene as target. Toyama et al. (1996) selected a pair of primers (MAR1 and MAR2; positions 190 to 206 and 1262 to 1278 respectively in the *Escherichia coli* 16S rRNA numbering system), flanking a 1088 base pair (bp) fragment, which could differentiate *T. maritimum* from the related species *Flavobacterium branchiophilum* and *F. columnare*, as well as from several other fish pathogenic bacteria. More recently, Bader & Shotts (1998) also selected a pair of *T. maritimum* species-specific PCR primers (Mar1 and Mar2; positions 77 to 98 and 456 to 476 respectively) from unique sequence stretches within this gene, delimiting a 400 bp DNA fragment.

However, despite the potential of PCR detection, these primers sets have not been tested simultaneously in order to compare their efficiency using a wide range of strains isolated from different species of marine fish and the environment. In the same way, neither Toyama et al. (1996) or Bader & Shotts (1998) determined the actual detection limits of each primer set with pure and mixed cultures, as well as in fish samples. Therefore, the aim of this study was to examine the specificity and sensitivity of the 2 PCR methods described previously for the identification of the *Tenacibaculum maritimum* strains. In addition, primers described by Toyama et al. (1996) were used to develop a nested PCR method for specific diagnosis of marine tenacibaculosis in fish samples.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in the primer specificity studies are listed in Tables 1 & 2. This collection comprises 76 *Tenacibaculum maritimum* strains isolated from 7 different fish species from 1995 to 2003 (Table 1) and 3 reference strains (NCIMB 2153, 2154^T and 2158) from the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) (Table 1). These belong to the main serotypes and clonal lineages described within this pathogen (Avendaño-Herrera et al. 2004a,b). For all experiments, these strains were routinely cultured on *Flexibacter maritimus* Medium (FMM) agar (Pazos et al. 1996) and incubated at 20°C for 72 h. Before the assay, all bacterial strains were confirmed as *T. maritimum* using biochemical tests and serological assays according to Avendaño-Herrera et al. (2004a). In addition, 5 reference strains of other species of the genus *Tenacibaculum*; *T. ovolyticum*, *T. mesophilum* and *T. amylolyticum* (NBRC 15947, 15992, 16307^T, 16308 and 16310^T), obtained from the NITE (National Institute of Technology and Evaluation) Biological Resource Center (Osaka, Japan), 27 strains of related filamentous bacterial species obtained from fish and seaweed and 21 pathogenic fish bacteria were also included as negative controls in the PCR (Table 2). Stock cultures were stored at –70°C in Cryo-bille tubes (AES Laboratory).

DNA extraction from pure and mixed bacterial culture. Chromosomal DNA was extracted using InstaGene Matrix (Bio-Rad) for pure bacterial cultures as previously described by Romalde et al. (1999). Briefly, *Tenacibaculum maritimum* and other non-*T. maritimum* bacterial species colonies were collected from the appropriate agar plates, suspended in 1 ml of sterile distilled water and centrifuged at 12 000 × *g* for 1 min. After the supernatants were removed, the pellets were resuspended in 200 µl of InstaGene Matrix and incubated at 56°C for 30 min. Then, the suspensions were mixed at high speed for 10 s and boiled in a water bath for 8 min. The lysates were mixed again at high speed and then were spun at 12 000 × *g* for 3 min. The concentration and quality of each DNA sample was examined spectrophotometrically at 260 nm. DNA from pure cultures was adjusted to a concentration of 30 ± 2 ng µl^{-1}. All DNA was maintained at −20°C until used for PCR reactions. All the experiments were carried out with DNA obtained in 3 different extractions for each bacterial strain.

PCR amplification. All PCR amplifications were performed with the commercial kit Ready-To-Go^{TM} PCR beads (Amersham Pharmacia Biotech), which included all the reagents needed for the PCR reactions (buffer, nucleotides and *Taq* DNA polymerase), with the exception of the specific primers and DNA template. Two species-specific primer pairs synthesized by Sigma-Genosys were used for the identification of *Tenacibaculum maritimum*: primers MAR1 (5'–AATGGCATCGTTTTAAA–3') and MAR2 (5'–C-GCTCTCTGTTGCCAGA–3') described by Toyama et al. (1996), and primers Mar1 (5'–TGTAGCTTGCTA-CAGATGA–3') and Mar2 (5'–AAATACCTACTCG-TAGGTACG–3') designed by Bader & Shotts (1998), which give amplification products of 1088 and 400 bp respectively. One µl of each DNA solution and 2 µmol of each primer were used in the amplification reaction. All samples were subjected to 40 cycles of amplification in 2 different thermal cyclers. Reaction mixtures (25 µl) were simultaneously amplified in T Gradient Thermocycler (Biometra) and Mastercycler personal (Eppendorf) apparatus. The amplification cycles used for denaturation, primer annealing to the template and primer extension were carried out according to each published PCR protocol. Negative controls, consisting of the same reaction mixture but with sterile distilled water instead of template DNA, were included in each batch of PCR reaction. The reproducibility of the results was assessed by repetition of the amplifications in at least 3 independent PCR assays.

Analysis of PCR products. Amplified products were detected by horizontal 1% (w/v) agarose gel electrophoresis for 60 min at 100 V in TAE 1× (0.04 M Tris, 0.0001 M EDTA, pH 8.0) electrophoresis buffer, visualized using 0.06 µg ml^{-1} of ethidium bromide (Bio-Rad) and photographed under UV light and computer digitised (Gel Doc 100, Bio-Rad). A 50 to 2000 bp ladder (Sigma Chemical) was used as a molecular mass marker. The presence of a single product of the appropriate size, identical to the reference strains, was considered as a positive result.

Restriction enzyme digestion. After the analysis of all PCR products, the isolates identified as *Tenacibaculum maritimum* with only one of the primer sets were subjected to restriction enzyme digestion. The MapDraw software from the Lasergene Navigator package (DNASTAR) was used to select the most adequate and specific restriction endonucleases for the 1088 bp amplification product. This selection was made on the basis of number, diversity and distribution of restriction fragments produced. Briefly, 2 µl of PCR product from each strain amplified by MAR1 and MAR2 was digested with 30 units of the restriction enzyme *Pvu*I or *Sac*II (Amersham Biosciences) according to the instructions of the manufacturer. Ten µl of the digested solution was analysed by horizontal electrophoresis. The restriction fragments of the PCR products from the reference strains were considered as positive controls.

Determination of PCR sensitivity from pure and mixed cultures. The detection limits of both species-specific primer sets were evaluated using bacterial suspensions of all reference strains. Pure colonies of *Tenacibaculum maritimum* were picked from FMM agar plates, adjusted to contain 10^9 cells ml^{-1} (McFarland Scale 4) and serially diluted in 0.85% sterile saline solution from 10^8 to 10 cells ml^{-1}. To determine the usefulness of the primer sets to amplify template DNA of *T. maritimum* from mixed cultures, bacterial suspensions of *Listonella anguillarum* (R82), *Photobacterium damselae* subsp. *piscicida* (TW 398), *Aeromonas salmonicida* (ATCC 14174) and *Lactococcus garvieae* (TW94W) were employed. Thus, bacterial mixtures of *T. maritimum* with 2 other different fish pathogens were prepared for PCR sensitivity testing. To each dilution of the *T. maritimum* strain, 100 µl of each bacterial suspension that contained approximately 10^7 cells ml^{-1} was added. Colony forming units (CFU) were estimated by plating each dilution onto appropriate media and counting the bacterial colonies produced.

All the dilutions of pure and mixed cultures were centrifuged at 12 000 × *g* for 2 min and washed twice with sterile distilled water to extract genomic DNA as before. Conditions for PCR amplification and electrophoresis were the same as described above.

Determination of PCR sensitivity from seeded fish samples. On the basis of the results obtained using bacterial cultures, PCR sensitivity was determined employing DNA extracted from *in vitro* seeded mucus, skin, kidney and blood, using the Toyama et al. (1996) protocol. All the tissue samples were taken from healthy *Tenacibaculum maritimum*-free juvenile turbot (12 g body weight). Mucus samples were obtained with a sterile 10 µl plastic loop or with a sterile scalpel blade by cutting the fish on the lateral line (Evans et al. 2001). The samples were then homogenized with 100 µl in phosphate buffered saline (PBS, pH 7.4) by repeated pipetting. In the case of skin and kidney, samples were collected using aseptic technique and divided into 500 and 30 mg pieces respectively. One hundred µl of blood samples were collected by vein puncture with a syringe and processed immediately. Each fish sample was seeded with 100 µl of the different dilutions of a culture suspension and homogenized for 60 s. The 3 reference strains of *T. maritimum* were tested in parallel. After incubation for 1 h, DNA extraction was performed with Dynabead® DNA DIRECT™ (Dynal) following the manufacturer's instruction, with the exception of blood samples, from which DNA was extracted as described above for bacterial culture. Non-inoculated fish samples, employed as negative controls, were processed in the same manner but with PBS instead of bacterial dilution. DNA obtained from the different tissues were maintained frozen at –20°C until used for PCR reactions. Conditions for PCR amplification and electrophoresis were the same as described above. In order to determine the reproducibility of results, all traits were assessed by repetition in at least 3 independent PCR assays, in which DNA obtained in 3 different extractions were employed.

Nested PCR amplification. To increase the sensitivity of the Toyama et al. (1996) PCR assay, a nested PCR was conducted on the whole dilution series from pure and mixed cultures, as well as infected tissues and blood with each reference strain. The first round of PCR was performed according to a previously described protocol (Edwards et al. 1989) with minor modifications. We used the same commercial reaction mixture and the universal primer pair pA (5'–AGAGTTTGATCCTGGCTCAG–3') and pH (5'–AAGGAGGTGATCCAGCCGCA–3') but the number of cycles was shortened to 25. These primers amplify the region from position 20 to position 1521 (*Escherichia coli* numbering system) of the 16S rRNA gene. After the first amplification by external primer was finished, 1 µl of each PCR product was used as template for the second amplification by the species-specific primer set MAR1 and MAR2. PCR samples were examined by gel electrophoresis and the samples were considered positive if the anticipated 1088 bp product was observed.

RESULTS

PCR amplification

To test the specificity of each primer pair on the identification of *Tenacibaculum maritimum*, DNA extracted from 79 strains of the target species as well as from 53 strains of other species were used for the PCR with each of the primer pairs. The MAR1–MAR2 primer pair identified all the *T. maritimum* strains studied, producing a unique and clear PCR product of the expected 1088 bp length. In contrast, the Mar1–Mar2 primer pair was capable of amplifying the 400 bp specific band from 76 of 79 strains of *T. maritimum* analyzed. The 3 *T. maritimum* strains (AZ202.1, AZ203.1 and PC438.1) not recognized by the Bader & Shotts (1998) pair of primers were isolated from sole (Table 1). Regardless of the primer set employed, amplification products were not obtained from the other bacterial species tested (Table 2).

When both PCR protocols were compared using 2 different thermal cyclers, no differences were detected in the results of the amplification products. In addition, reproducibility of PCR results was demonstrated since the same results were obtained in at least 3 independent PCR assays for each primer set tested.

Restriction enzyme digestion

To verify if the 3 isolates from sole only recognized using the Toyama et al. (1996) primer set belonged to *Tenacibaculum maritimum* species, 2 endonucleases (*Pvu*I and *Sac*II) were selected as the most adequate to confirm the specificity of the amplified fragment. The

Table 1. *Tenacibaculum maritimum* strains used in this study and the results from the specific single PCR detection methods employed. NCIMB: National Collection of Marine and Industrial Bacteria (Aberdeen, UK)

Type of samples	Isolation source	No. of strains	No. of strains identified with sets MAR1–MAR2	 Mar 1–Mar2
Strains isolated from diseased fish (kidney and/or skin)	*Scophthalmus maximus*	49	49	49
	Solea senegalensis	17	17	14
	Solea solea	1	1	1
	Sparus aurata	5	5	5
	Salmo salar	1	1	1
	Seriola quinqueradiata	1	1	1
	Dicentrarchus labrax	2	2	2
Reference strains				
T. maritimum NCIMB2153	*Acanthopagrus schlegeli*	1	1	1
T. maritimum NCIMB 2154^T	*Pagrus major*	1	1	1
T. maritimum NCIMB 2158	*Solea solea*	1	1	1

Table 2. Bacteria from other species included in this study as negative controls in the PCR analysis. NBRC: NITE Biological Resource Center (Osaka, Japan); ATCC: American Type Culture Collection (Rockville, USA); NCIMB: National Collection of Industrial and Marine Bacteria (Aberdeen, UK). No amplification by PCR was detected with either primer set tested. Numbers in parentheses indicate the number of strains assayed

Bacterial strains	Source
Tenacibaculum ovolyticum NBRC 15947	NBRC
Tenacibaculum ovolyticum NBRC 15992	NBRC
Tenacibaculum mesophilum NBRC 16307^T	NBRC
Tenacibaculum mesophilum NBRC 16308	NBRC
Tenacibaculum amylolyticum NBRC 16310^T	NBRC
Flexibacter-like isolated from (17) diseased fish (kidney and/or skin)	Laboratory collection
Flexibacter-like isolated from seaweed (6)	Laboratory collection
Flavobacterium sp. (4)	Laboratory collection
Flavobacterium psychrophilum PT41	Laboratory collection
Pseudomonas fluorescens ATCC 13525^T	ATCC
Pseudomonas aeruginosa ATCC 27853	ATCC
Aeromonas salmonicida ATCC 14174	ATCC
Aeromonas media ATCC 33907^T	ATCC
Alcaligenes pacificus ATCC 27122	ATCC
Vibrio ordalii NCIMB 2107	NCIMB
Streptococcus parauberis RA9	Laboratory collection
Lactococcus garvieae TW94W	Laboratory collection
Streptococcus iniae 990	Laboratory collection
Pseudomonas anguilliseptica 899	Laboratory collection
Renibacterium salmoninarum ATCC 33209^T	ATCC
Yersinia ruckeri TWS30	Laboratory collection
Photobacterium damselae subsp. *piscicida* TW 398	Laboratory collection
Listonella anguillarum R82	Laboratory collection
Vibrio harveyi TW425	Laboratory collection
Vibrio splendidus I PC400.1	Laboratory collection
Vibrio alginolyticus ATCC 17749^T	ATCC
Vibrio vulnificus A11	Laboratory collection
Vibrio pelagius I PC376.1	Laboratory collection
Vibrio pelagius II RQ167.1	Laboratory collection

restriction profiles of the PCR products obtained with the Toyama et al. (1996) primers, including the isolates not recognized as *T. maritimum* by Mar1 and Mar2 were identical for all the strains tested (Fig. 1), and coincident with the expected sizes calculated using the MapDraw software. Thus, endonuclease *Pvu*I rendered 2 fragments of 400 and 688 bp, while the enzyme *Sac*II produced 2 bands of 336 and 752 bp.

Detection limit for the primer pairs

The sensitivity and robustness of each species-specific PCR primer set were determined by amplification of DNA extracted from different bacterial suspensions (10^8 to 10 cells ml^{-1}) of 3 *Tenacibaculum. maritimum* reference strains (Table 3). When the MAR1–MAR2 primer pair was tested, the expected 1088 bp PCR product was obtained with samples containing as low as 80 to 500 cells per PCR tube (Fig. 2A). With respect to Mar1–Mar2 primer pair, a fragment of the expected size (400 bp) was also amplified, allowing the detection of 8×10^2 to 5×10^3 *T. maritimum* cells per PCR tube (Fig. 2B).

When DNA extracted from mixed cultures were used as template in single PCR amplification, the limits of detection of the *Tenacibaculum maritimum* with the 2 primer sets were identical to the level of amplification obtained with DNA from pure culture of reference strains (Table 3). Therefore, the other species of fish pathogens assayed did not interfere in the PCR sensitivity, even when high concentrations of non-*T. maritimum* strains were employed ($4.0 \pm 1 \times 10^6$ CFU ml^{-1}).

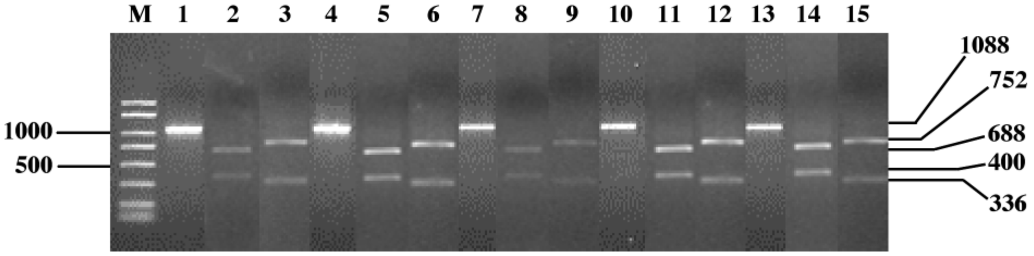

Fig. 1. Specific PCR products obtained with MAR1 and MAR2 (lanes 1, 4, 7, 10, 13) and digestion of the amplified product with the restriction enzymes *Pvu*I (lanes 2, 5, 8, 11, 14) and *Sac*II (lanes 3, 6, 9, 12, 15). Lanes: M: AmpliSize Molecular Ruler (50–2000bp ladder, Sigma); 1–3: *Tenacibaculum maritimum* reference strain NCIMB 2153; 4–6: *Tenacibaculum maritimum* reference strain NCIMB 2154^T; 7–9: sole isolate AZ202.1; 10–12: sole isolate AZ203.1; 13–15: sole isolate PC438.1. Numbers on the left indicate the position of molecular size marker in bp. Numbers on the right indicate the size of the specific amplicons and the endonuclease restriction products in bp

Table 3. Sensitivity obtained with the different PCR protocols evaluated in single- and nested-PCR using pure and mixed cultures, as well as fish samples seeded with *Tenacibaculum maritimum*. CFU: colony forming units; NA: no amplification product; NT: not tested

	MAR1–MAR2				Mar1–Mar2	
	Single PCR		Nested-PCR		Single PCR	
	Cells tube^{-1}	CFU ml^{-1} or CFU g^{-1}	Cells tube^{-1}	CFU ml^{-1} or CFU g^{-1}	Cells tube^{-1}	CFU ml^{-1} or CFU g^{-1}
Cultures						
Pure	80–500[a]	1.6×10^4–1.1×10^5	1–5	1.6×10^2–1.1×10^3	8×10^2–5×10^3	1.6×10^5–1.1×10^6
Mixed	80–500	1.6×10^4–1.1×10^5	1–5	1.6×10^2–1.1×10^3	8×10^2–5×10^3	1.6×10^5–1.1×10^6
Fish samples						
Mucus	$1–2 \times 10^4$	$5–6 \times 10^6$	1–3	$5–6 \times 10^3$	NT	NT
Skin	160–250	$1–1.5 \times 10^4$	1–3	$1–1.5 \times 10^2$	NT	NT
Kidney	NA	NA	160–250	$1.7–2.3 \times 10^5$	NT	NT
Blood	$1–3 \times 10^4$	$2–4 \times 10^6$	5100–200	$2–4 \times 10^4$	NT	NT

[a] The values correspond to the amplification range of the 3 reference strains in triplicate

PCR sensitivity from infected fish samples and blood

With the knowledge that the Toyama et al. (1996) protocol allowed the confirmation of all *Tenacibaculum maritimum* strains examined and due to the best sensitivity achieved with this protocol in pure and mixed cultures, only the primer pair MAR1–MAR2 was tested with DNA extracted from the experimentally seeded fish tissues and blood. Application of the PCR protocol described above showed that *T. maritimum* could be detected in mucus, skin and blood at a detection level on the order of 10^2 to 10^4 cells per reaction. However, no amplification product was observed when DNA samples of kidney were tested (Table 3). When the tissues used as negative controls were tested with the same primers, no amplification occurred. Finally, the total time for the procedure, including DNA extraction from samples, amplification and gel electrophoresis, was shorter than 7 h.

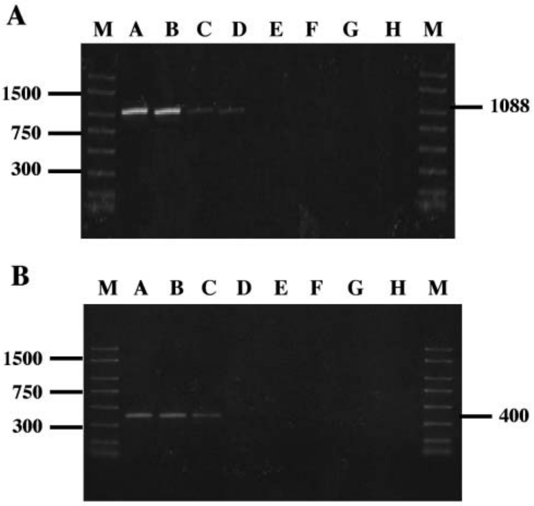

Fig. 2. Sensitivity of PCR protocol using (A) MAR1–MAR2 and (B) Mar1– Mar2 primer sets. Lanes M: AmpliSize Molecular Ruler (50 to 2000 bp ladder, Sigma); Lanes 1 to 8: serial decimal dilutions of the chromosomal DNA extracted from reference strain NCIMB 2154^T, ranging from 4×10^8 (Lane 1) to 4×10^1 (Lane 8) cells ml^{-1}. Numbers on the left indicate the position of molecular size marker in bp. Numbers on the right indicate the size of the specific amplified products in bp

Nested PCR amplification

When 1 µl of the first round product amplified with the universal primer set was used as a template for species-specific primer in nested PCR assays, the detection limit of all assays decreased by approximately 2 to 3 orders of magnitude compared with conventional PCR assay, regardless of the type of samples employed (Table 3). Then, when pure and mixed cultured *Tenacibaculum maritimum* were used in DNA extraction and nested PCR amplification, the detection limit achieved was about 1 to 5 bacteria per PCR tube (which corresponds to 1.6×10^2 to 1.1×10^3 CFU ml^{-1}). In the case of DNA extracted from mucus and skin samples the nested PCR methods allowed the detection of approximately 1 to 3 *T. maritimum* cell per PCR tube (Fig. 3), and showed a reduction of sensitivity in 1 or 2 log-units for the blood samples. Although the single PCR did not yield amplification products in kidney samples, the nested PCR produced a unique and clear fragment of the expected size (1088 bp) when using this tissue, a sensitivity of approximately 2 ×

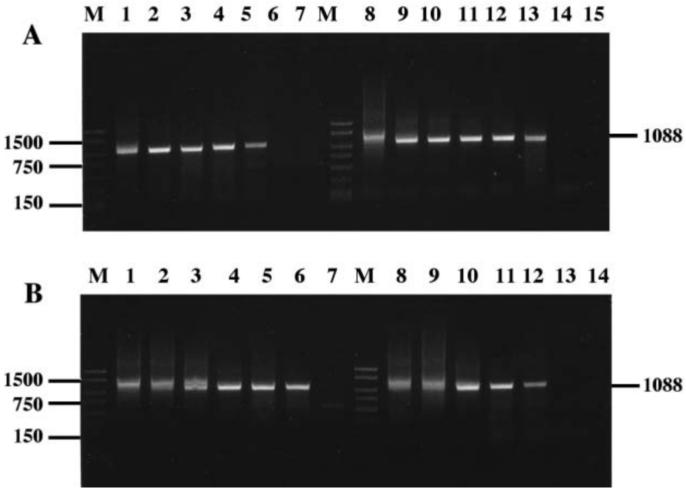

Fig. 3. Detection by nested PCR of *Tenacibaculum maritimum* in turbot tissues seeded with decimal dilutions of the reference strain NCIMB 2154^T. Tissues evaluated were (A) blood and mucus and (B) skin and kidney. Lanes in A: M: AmpliSize Molecular Ruler (50 to 2000 bp ladder, Sigma); 1 to 7: DNA extracted from blood seeded with decimal dilutions ranging from 4×10^8 (Lane 1) to 4×10^2 (Lane 7) cells ml^{-1}; 8–14: DNA extracted from mucus seeded with decimal dilutions ranging from 4×10^8 (Lane 8) to 4×10^2 (Lane 15) cells ml^{-1}; 15, negative control (no DNA). Lanes in B: M: AmpliSize Molecular Ruler (50 to 2000 bp ladder, Sigma); 1 to 7: DNA extracted from skin seeded with decimal dilutions ranging from 4×10^8 (Lane 1) to 4×10^2 (Lane 7) cells ml^{-1}; 8 to 14: DNA extracted from kidney seeded with decimal dilutions ranging from 4×10^8 (Lane 8) to 4×10^2 (Lane 14) cells ml^{-1}. Numbers on the left indicate the position of molecular size marker in bp. Numbers on the right indicate the size of the specific amplified products in bp

10^5 CFU g^{-1} fish tissue being observed. DNA extracted from fish samples used as negative controls did not yield any amplification product. The total procedure, from the point of DNA extraction to observation in an agarose gel, was increased to 9 h with the second round of PCR.

DISCUSSION

One of the most critical steps in the study of bacterial fish diseases is the correct identification of the infectious agent. Rapid diagnosis of outbreaks is essential for an effective control, but current microbiological methods based on culture and biochemical characterization are time-consuming. To overcome these difficulties, DNA-based methods have been developed in recent years for the fast and sensitive diagnosis of the bacterial pathogens in aquatic species. In the case of *Tenacibaculum maritimum*, Toyama et al. (1996) and Bader & Shotts (1998) used the 16S rRNA gene sequence available in public databases for the design of specific primers to be employed in the PCR. However, until now the specificity and sensitivity of these primer sets have not been evaluated in parallel in order to propose the best PCR protocol to detect this pathogen in research laboratories.

Comparing the specificity of the 2 PCR protocols, the sequence of both primer pairs were species-specific for *Tenacibaculum maritimum*, and no amplification products were obtained from chromosomal DNA of other non-*T. maritimum* bacteria tested. These results agree with those reported by the designers of both sets of primers (Toyama et al. 1996, Bader & Shotts 1998). However, when all bacteria listed in Table 1 were screened, the number of strains identified by each primer pair was different. Whereas all the isolates rendered the specific amplicon when the MAR1–MAR2 (Toyama et al. 1996) primer pair was used, the Mar1–Mar2 (Bader & Shotts 1998) primer set failed to identify 3 isolates. However, the digestion patterns of the MAR1–MAR2 amplified products of 1088 bp with the restriction enzymes *Pvu*I and *Sac*II allowed us to confirm the assignation of all the strains to the *T. maritimum* species.

Recently, Cepeda et al. (2003) also employed the Mar1–Mar2 primer pair, making modifications to the PCR programme described by Bader & Shotts (1998) in order to reduce the time to achieve the diagnosis of *Tenacibaculum maritimum*. However, when we used this protocol with pure cultures of *T. maritimum* strains using the same extraction system and identical conditions as described by these authors, none of isolates tested generated an amplification signal (data not shown). This failure should probably be attributed to the extremely short time (2 s) employed in the annealing step, although we can not rule out the influence of other variables which theoretically could alter the test performance, such as source of reagents or disposable supplies.

In the present study, the sensitivity of both PCR detection methods was also different. It has been reported that sensitivity of PCR varies depending on the size of amplicons, and generally primers yielding smaller amplicons will be able to detect lower levels of target organisms (Otta et al. 1999, Hossain et al. 2001). However, our results using either pure or mixed cultures showed a reduction of sensitivity in at least one order of magnitude of the Mar1–Mar2 primer pair in comparison with MAR1–MAR2, although the former primer set yield a shorter amplicon (approximately 700 bp) than the latter one.

Our study has shown that when the single PCR protocol of Toyama et al. (1996) was used with DNA extracted from seeded mucus, skin tissue and blood samples the sensitivity level was 10- to 100-fold lower than that observed with pure and mixed cultures, probably due to presence of host DNA and undefined inhibitors of PCR (Wilson 1997). This level of sensitivity is probably sufficient to detect *Tenacibaculum maritimum* acute infections in fish, but not when the pathogen is present in very low numbers in asymptomatic or carrier fish.

A nested PCR approach greatly increases the sensitivity of detection, and can be useful for the detection of very low copy numbers of template (Osorio & Toranzo 2002). In our case the nested PCR assay supports this fact since an increase in sensitivity of at least 100 times was observed compared to conventional PCR; this sensitivity is in the same range as those achieved for other bacterial fish pathogens (Osorio et al. 1999, Taylor & Winton 2002). Although, we found that all tissues sampled could be used to detect the bacterium in a nested PCR protocol, negative results were obtained when using DNA extracted from kidney samples in a single PCR. Similar problems in detecting other fish pathogens in kidney have been attributed to PCR inhibitors (Chase & Pascho 1998). The tissues that allowed detection of *Tenacibaculum maritimum* most easily were the skin and mucus with detection limits of about to 10^2 CFU g^{-1} to 10^3 CFU ml^{-1} respectively. This finding is in agreement with the observation previously reported for *F. columnare* by Bader et al. (2003). Therefore, the use of nested PCR with the MAR1–MAR2 primer set in mucus samples becomes a useful, rapid and non-destructive procedure for the detection of *T. maritimum* in carrier fish.

In summary, on the basis of the results obtained in this work we propose the Toyama et al. (1996) PCR procedure as the most adequate for an accurate detection of *Tenacibaculum maritimum* in diagnostic pathology as well as in epidemiological studies of gliding bacterial disease of marine fish. In addition, the nested PCR provides at least 100 times more sensitivity than conventional PCR for the detection of *T. maritimum* not only in pure and mixed bacterial cultures, but also in experimentally seeded fish tissues. The accuracy of this method is currently being investigated with natural samples from different fish farms in order to evaluate its efficacy, under field conditions, in detecting the pathogen in diseased and asymptomatic carrier fish.

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A non-destructive method for rapid detection of *Tenacibaculum maritimum* in farmed fish using nested PCR amplification

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Abstract

Marine tenacibaculosis caused by *Tenacibaculum maritimum* is an important disease in different cultured marine fish. Some of the presently available techniques for the detection of *T. maritimum* are either time consuming or lack sufficient sensitivity. In this study, we evaluated the efficacy of a nested PCR method for the detection of *T. maritimum* using non-destructive mucus samples from different cultured species of marine fish. To test this method sixty asymptomatic and symptomatic cultured fish were examined. The nested PCR assay allowed the detection of *T. maritimum* in 100% of mucus samples from farmed turbot, sole and gilthead seabream that were undergoing an epizootic of tenacibaculosis. Only 81% of the mucus samples from these diseased fish were positive by plating methods. When the nested PCR protocol was applied to different mucus samples obtained from apparently healthy fish, the detection of *T. maritimum* was positive in 15 of 29 fish tested. Nine of these fish had been diagnosed as negative for the presence of *T. maritimum* by cultivation on agar. The method presented here demonstrated that non-lethal mucus samples can be used with screening purpose and early diagnosis of marine tenacibaculosis, allowing the rapid detection of *T. maritimum* (in only 7 h) without previous isolation in culture media.

Introduction

Cultured fish are susceptible to a wide range of bacterial infections, and losses associated with the diseases subsequently make a significant impact on the quality and volume of fish produced worldwide. *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) is one of these threatening microorganisms, being the causative agent of tenacibaculosis or flexibacteriosis in a great variety of valuable marine fish species in Japan, North America, several countries in Europe and Australia (Hikida et al., 1979; McVicar & White, 1979; Devesa et al., 1989; Bernardet et al., 1990; Alsina & Blanch, 1993; Handlinger et al., 1997; Cepeda & Santos, 2002; Avendaño-Herrera et al., 2004a).

The disease is currently diagnosed by the clinical signs of the affected fish, especially severe necrotic lesions on the body surface, skin ulcers, jaw erosion, frayed fins and tail rot (Campbell & Buswell, 1982; Devesa et al., 1989; Ostland et al., 1999). However, the isolation of *T. maritimum* from fish tissues can be very difficult, due to its extremely slow growth on synthetic specific media, where the pathogen is normally overgrown by many of the species coexisting in external samples. These facts, makes it very difficult to visualize and isolate *T. maritimum*.

Rapid detection and identification of this pathogen is crucial for effective management and disease control in sea farming. Initial attempts to detect *T. maritimum* using molecular tools were based on the design of two pairs of oligonucleotides primers for the polymerase chain reaction (PCR)-based method using the 16S rRNA gene sequence as target (Toyama et al., 1996; Bader & Shotts, 1998). Cepeda et al. (2003) published a nested PCR system for the detection of *T. maritimum* in fish tissues based on modifications of the PCR programme described previously by Bader & Shotts (1998) in order to reduce the time to achieve the diagnosis of this bacterium. However, when we used this protocol in the same conditions described by these authors with pure cultures of *T. maritimum* strains, none of the isolates tested generated an amplification signal (Avendaño-Herrera et al., 2004b).

On the other hand, we also compared the specificity and sensitivity of these two primer pairs, finding the Toyama PCR procedure the most adequate for an accurate detection of *T. maritimum* in diagnostic pathology as well as in epidemiological studies of marine tenacibaculosis (Avendaño-Herrera et al., 2004b). Although this method proved to be useful to detect *T. maritimum* acute infections in fish, the level of sensitivity was not sufficient to detect the pathogen when present in very low number in asymtomatic or carrier fish. In order to increase its sensitivity we developed a nested PCR approach, which was evaluated experimentally by seeded fish tissues, including mucus, skin, kidney and blood (Avendaño-Herrera et al., 2004b). With this method, we found an easy detection of *T. maritimum* in mucus samples with a level of sensitivity of about 10^3 CFU ml^{-1}. However, despite its potential, no application of this nested PCR protocol with mucus samples from cultivated fish was performed.

Therefore, in this study we evaluated the efficacy of a nested PCR method for the detection of *T. maritimum* using non-destructive mucus samples from different cultured species of marine fish.

Materials and methods

Fish samples

To evaluate the possible usefulness of the nested PCR method to detect *T. maritimum* in non-destructive samples, a total of sixty fish ranging from 10 to 300 g were obtained from turbot, sole and gilthead seabream farmed in the northwest of Spain and Portugal. Fish were sent from fish farms to the laboratory of the University of Santiago de Compostela (Spain) where they were subjected to bacteriological examination (agar cultivation and biochemical tests) and analysed by nested PCR. Fish were sent on ice and analysed within 5 h of capture. To facilitate our study, the species examined were classified into two groups: symptomatic (n = 31) and asymptomatic (n = 29) fish. The first group was defined as fish with the main signs of the diseases such as eroded mouths, skin ulcers and rotten, frayed fins, while the second group compiled fish that did not show the clinical signs of marine tenacibaculosis (Table 1).

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		Detection of *T. maritimum* by		
Source	**No. of samples**	**Nested PCR**	**Culture**	**Microscopy**
Symptomatic fish				
Turbot (*Scophthalmus maximus*)	21	21	16	21
Sole (*Solea senegalensis*)	6	6	6	6
Gilthead seabream (*Sparus auratus*)	4	4	3	4
Asymptomatic fish				
Turbot (*Scophthalmus maximus*)	29	15	6 *	9 *

[*] Samples also positive by nested PCR.

Table 1. Detection of *T. maritimum* in skin mucus samples from cultured marine fish, using nested PCR, agar cultivation and microscope observation.

Collection of the mucus samples

Mucus samples from fish were obtained by gently passing a sterile 10 µl plastic inoculating loop or a sterile scalpel blade along the lateral surface of individual fish (Evans et al., 2001). The mucus samples were then diluted 1:10 (v/v) in 100 ml sterile phosphate buffered saline (PBS, pH 7.4) and maintained frozen at –20°C until they were used for DNA extraction.

Microscopical and bacteriological analysis

In order to compare the efficacy of the nested PCR procedure, other standard conventional methods were carried out. For microscope observations, smear from skin samples were examined using a light microscope at 400 x magnification. At the same time, samples from skin were directly streaked onto plates containing *Flexibacter maritimus* Medium (FMM) agar (Pazos et al., 1996), which were incubated at 20°C for 72 h. After this, the colonies that exhibited the typical features for this species: flat, pale-yellow with uneven edges and strong adherence to the medium were chosen for isolation and subjected to standard phenotypical tests (Avendaño-Herrera et al. 2004a). Stock cultures of the strains were stored frozen at –70°C in Criobille tubes (AES Laboratory, France). Simultaneously, pure cultures of *T. maritimum* were scraped off the plates, resuspended in 1 ml of sterile distilled water, and the samples were maintained at –20°C until the DNA extraction.

DNA extraction

DNA was extracted using 2 different commercial systems: InstaGene Matrix (Bio-Rad, Madrid, Spain) for the pure cultures, and Dynabeads DNA DIRECT™ (Dynal) for mucus samples. In all cases, DNA purification was performed according to the manufacturer's instructions. One ml of each DNA extracted was used directly for single and nested PCR amplification and the remaining DNA sample was maintained at –20°C. All extractions included at least two positive and negative controls. The positive controls consisted of 10^5 cells from a pure culture of the *T. maritimum* strain NCIMB 2154^T and mucus seeded with the same concentration of this reference strain. Sterile distilled water and non-inoculated mucus samples were employed as negative controls.

Nested PCR analysis

In this study a nested PCR method recently developed in our laboratory was applied to the mucus samples (Avendaño-Herrera et al., 2004b). The first round of PCR was performed according to a previously described protocol by Edwards et al., (1989) with minor modifications. This method utilizes the universal primer pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′), but the reaction condition was shortened to 25 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 90 s and in the final cycle samples were incubated at 72°C for 5 min. A preheating cycle at 95°C for 4 min was included. All PCR amplifications were done using the commercial kit Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech, Barcelona, Spain), which included all the reagents needed for the PCR reactions (buffer, nucleotides and *Taq* DNA polymerase), with the exception of the specific primers and DNA template. The reaction mixtures were amplified in a Mastercycler personal thermal cycler (Eppendorf, Hamburg, Germany). After the first amplification by the external primers was finished, 1 µl of each PCR product was used as template of the second amplification by the species-specific primer set MAR1 (5′-AATGGCATCGTTTTAAA-3′) and MAR2 (5′-CGCTCTCTGTTGCCAGA-3′) (Toyama et al., 1996). The samples were denatured at 94°C for 2 min, followed by 40 cycles of 94°C for 2 min, 45°C for 90 s, and 72°C for 2 min. The tubes were then held at 4°C. DNA's extracted from negative and positive controls were included in each batch of PCR reaction. Single PCR analysis was used for the detection of *T. maritimum* from pure cultures, the conditions for PCR amplification were the same as described for the second amplification.

Analysis of PCR products

Amplified products were detected by horizontal 1% (w/v) agarose gel electrophoresis for 60 min at 100 V in TAE 1X (0.04 M Tris, 0.0001M EDTA, pH 8.0) electrophoresis buffer, visualized using 0.06 µg ml^{-1} of ethidium bromide (Bio-Rad) and photographed under UV light and computer digitized (Gel Doc 100, Bio-Rad). A 50-2000-bp ladder (Sigma Chemical Co, St. Louis, MO, USA) was used as a molecular size marker. The presence of a single band of 1088 base pair (bp) was considered as a positive result.

Results and discussion

The need for fast, specific and sensitive molecular tools for the detection of important bacterial pathogens in cultivated marine fish using non-destructive samples is particularly crucial in the context of sea-farming (Arias et al., 1995; Osorio et al., 1999; Bader et al., 2003).

In this work, we tested whether *T. maritimum* could be detected directly in field conditions utilizing skin mucus as a non-destructive sampling procedure by using a nested PCR assay. The application of this protocol gave positive amplification to *T. maritimum* for mucus samples obtained from all symptomatic fish tested in this study, regardless of the fish host (Table 1). All of them rendered a unique and clear PCR band of the expected 1088 bp length (Figure 1),

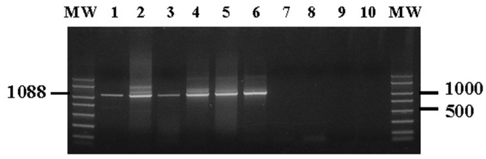

Figure 1. Detection of *T. maritimum* from mucus samples obtained from symptomatic (lanes 2, 4, and 5) and asymptomatic (lanes 3, 6, 7, 8 and 9) fish by nested PCR. Lanes: MW, AmpliSize Molecular Ruler (50-2000-bp ladder, Sigma); 1, positive control (DNA extracted from mucus seeded with *Tenacibaculum maritimum* NCIMB 2154^{T}); 2 to 9, DNAs extracted from mucus samples; 10, negative control (no DNA). Numbers on the right indicate the position of molecular size marker in kb. Numbers on the left indicate the size of the specific amplified products in bp.

while the negative control did not yield any amplification product. Microscopic examination of smears from skin lesions of these symptomatic fish revealed the presence of abundant long, thin, rod-shaped bacteria with the ability to flex. However, the microbiological analysis on FMM plates of external skin failed in the recovery of this bacterium in 1 and 5 samples of gilthead seabream and turbot respectively (Table 1). This failure can be explained by the growth inhibition of *T. maritimum* by other bacterial species present in the samples (Pazos et al., 1996).

When the nested PCR protocol was applied to different mucus samples obtained from apparently healthy fish, the detection of *T. maritimum* was positive in 15 of 29 asymptomatic fish tested (Table 1), yielding the expected 1088 bp fragment. In the other fish as well as in the negative controls no amplification was obtained. When all fish from this group were examined by microscopy, a few gliding bacteria were observed in skin scraped over large areas of the body in only 9 fish. Attempts to culture this gliding bacterium from the body surface was successful in 6 of the 29 cases analyzed, and the remaining fish were diagnosed as negative for the presence of *T. maritimum* by classical plate-culturing methods. It is important to note that all of the samples, which were positive by microscopical and bacteriological analysis, were also positive in the nested PCR.

The results presented in this work demonstrated clearly that the nested PCR applied to mucus samples from symptomatic fish was effective for the rapid detection of *T. maritimum* in the field, avoiding the possible false negative results obtained when only plate culture procedure is employed. These results are in agreement with the observation previously reported by Avendaño-Herrera et al., (2004b) when experimentally seeded fish tissues were employed. In the case of asymptomatic fish, it is important to note that the positive results reflected a high probability of infection, because a few days post-analysis these groups of fish showed disease signs followed by mortalities in the farms.

The effectiveness of the use of mucus samples for rapid diagnosis of marine tenacibaculosis could be explained by the primary site of infection of the pathogen, which is the body surface. In fact, it has been demonstrated that the skin mucus does not contain compound that inhibit the growth of *T. maritimum*, and therefore the localization of the bacteria is more likely within the mucus layer (Magariños et al., 1995; Handlinger et al., 1997).

In summary, from a practical point of view, we consider that the nested PCR approach is very useful for mucus screening purpose and, therefore, one of the strongest tools for early diagnosis of marine tenacibaculosis with a positive economic impact on marine fish culture. This non-destructive method allows the rapid detection of *T. maritimum* (in only 7 h) without previous isolation in culture media. This protocol can be useful for further understanding of the epidemiology of *T. maritimum* in order to elucidate possible transmission routes.

Acknowledgement

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VI. SUPERVIVENCIA DE *Tenacibaculum maritimum* EN EL MEDIO ACUÁTICO

VI. A. PUBLICACIONES

Artículo nº 6: Use of microcosm to determine the survival of the fish pathogen *Tenacibaculum maritimum* in seawater. **Environmental Microbiology** (in press).

CONTENIDOS:

- Preparación de los microcosmos.
- Recuento de bacterias cultivables y totales.
- Detección del patógeno mediante la técnica de PCR, convencional y anidada.
- Análisis de los componentes de la superficie celular y de la forma bacteriana.
- Estudios de reactivación de *Tenacibaculum maritimum*.
- Ensayos de actividad inhibitoria usando las bacterias autóctonas aisladas del medio acuático contra *Tenacibaculum maritimum*.

Use of microcosms to determine the survival of the fish pathogen *Tenacibaculum maritimum* in seawater

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Summary

The survival of the fish pathogen *Tenacibaculum maritimum* in different seawater microcosms was investigated during 160 days. The persistence of culturable cells was greater in sterile than in natural seawater. Standard plate counts showed that *T. maritimum* survived in sterile seawater for more than 5 months at concentration around 10^3 CFU ml^{-1}. However, *T. maritimum* proved to be very labile in non-sterile seawater, rendering culturable cells no longer than 5 days. These results were confirmed when DNA-based methods were applied. Regardless of the microcosms used, epifluorescence microscopy counts remained at about 10^6 cells ml^{-1} throughout the experiment, even though we can not distinguish *T. maritimum* in the case of non-sterile microcosms. Resuscitation assays with addition of fresh medium to non-sterile microcosms did not favour the recovery of *T. maritimum* on solid media. Although morphological changes from filamentous to spheres were observed after 3 days in the non-sterile microcosms, in the case of the sterile microcosms this change was observed at the sixth day. The biochemical, physiological, serological and genetic characteristics were unaffected in the sterile microcosms. The overall results contribute to a better understanding of the behaviour of *T. maritimum* in natural seawater and suggest that the aquatic bacterial population play an important role in the survival of this fish pathogen.

Key words: *Tenacibaculum maritimum*, survival, fish pathogen, seawater microcosms.

Introduction

Marine tenacibaculosis is a serious bacterial disease affecting a great variety of valuable cultured fish species, as well as wild fish, in Japan, North America, several countries in Europe and Australia (Hikida *et al*., 1979; Baxa *et al*., 1986; Devesa *et al*., 1989; Bernardet *et al*., 1990; 1994; Chen *et* al., 1995; Handlinger *et al*., 1997; Ostland *et al*., 1999; Cepeda and Santos, 2002; Avendaño-Herrera *et al*., 2004a). The aetiological agent of this disease is *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (Suzuki *et al*., 2001), a Gram negative and filamentous bacterium, which directly attacks the body surface of fish (Magariños *et al*., 1995), causing ulcerative skin lesions, necrosis, eroded and hemorrhagic mouth, frayed fins and tail rot (McVicar and White, 1979; Campbell and Buswell, 1982; Baxa *et al*., 1986; Devesa *et al*., 1989).

Although the phenotypic, antigenic and molecular characteristics of *T. maritimum* have been examined by several authors (Wakabayashi *et al*., 1984; Bernardet and Grimont, 1989; Bernardet *et al*., 1990; Chen *et* al., 1995; Ostland *et al*., 1999; Avendaño-Herrera *et al*., 2004a and b), to our knowledge no studies of the behaviour of this bacterium in the aquatic environment have been performed and, therefore, the possible role of water as a route of transmission of the disease is still unknown.

Numerous studies describe *T. maritimum* as a fastidious organism, and problems with culturing the bacterium have been reported (Pazos *et al*., 1996; Avendaño-Herrera *et al*., 2004c and d). Its ability to survive and remain infective in the aquatic environment has not yet been elucidated and must be taken into account as a major determinant in the spread of the disease. On the other hand, under adverse conditions, it is possible that the bacterium enters a ‘dormant’ or viable but non-culturable state (VBNC), as has been well documented for many other fish pathogens, such as *Vibrio vulnificus*, *Photobacterium damselae* subsp. *piscicida*, *Streptococcus parauberis* and

Flavobacterium psychrophilum (Oliver *et al*., 1991; Magariños *et al*., 1994; Currás *et al*., 2002; Madetoja *et al*., 2003). These bacteria lose their ability to grow on solid and liquid media, nonetheless, retain viability, being the VBNC state considered a survival strategy common to numerous bacterial species (Colwell and Grimes, 2000). Therefore, if *T. maritimum* enters a VBNC state, it may constitute a potential risk for fish farms and perhaps could explain how tenacibaculosis outbreaks can occur in fish populations which apparently have not come into contact with the pathogen.

The main objective of this research was to compare the survival behaviour of *T. maritimum* in seawater microcosms under sterile and natural conditions. Changes in the phenotypic and genetic characteristics of this fish pathogen, its capacity to enter a 'dormant' state, as well as the influence of autochthonous aquatic microbiota in its survival dynamics were also evaluated.

Results

Long-term survival of T. maritimum *in sterile microcosms*

The survival capacity of the *T. maritimum* strain, inoculated into microcosms with sterile (filtered and autoclaved) seawater is shown in Fig. 1. In general, on the second day of the experiment the number of culturable bacteria increased by 1 log-unit, and then a constant reduction of approximately 4 log-unit in CFU ml^{-1} was detected until day 40 from an initial inoculum of 10^7 cells ml^{-1}. After this, standard plate counts showed a slight increase of culturable *T. maritimum* followed by a stabilization around 10^3 CFU ml^{-1} until the end of the experiment. By epifluorescence microscopy, although some variations lower than 1 log-unit were observed during the first 60 days, from this point total counts of *T. maritimum* cells remained nearly constant during the experimental period, at values of approximately 10^6 bacteria ml^{-1} (Fig. 1).

Application of single and nested PCR protocols gave positive amplification of *T. maritimum* for all water samples obtained from sterile microcosms during the study period. All rendered a unique and clear PCR band of the expected 1088 bp length, while sterile microcosms without addition of *T. maritimum*, as expected, did not yield any amplification product (data not shown).

Survival of T. maritimum *in non-sterile seawater microcosms*

A slight increase in number of culturable *T. maritimum* was observed for all non-sterile water microcosms during the first 2 days. Then, standard plate counts showed that the number of culturable *T. maritimum* declined very rapidly, with no cells being detectable 5 days after the beginning of the experiment (Fig. 2). In contrast, culturability of the autochthonous populations of marine bacteria in the non-sterile water remained relatively constant, varying by 1 log-unit throughout the experimental period. This observation was supported by results of epifluorescence microscopy (Fig. 2).

The results obtained from analysis of the microcosms using single PCR gave positive reactions only during the first 4 days of the experiment (Fig. 3 A). However, when nested PCR was performed on DNA extracted from non-sterile water, the expected fragment for *T. maritimum* (1088 bp) was detected in samples collected from this microcosms until 10 days from the onset of the study (Fig. 3 B). After this period, no positive results were recovered even when nested PCR was performed with DNA extracted from the remaining volume of the non-sterile seawater microcosms.

Morphological, biochemical, antigenic and genetic changes

Microscopic observations demonstrated remarkable changes in morphology and size of the *T. maritimum* cells after the three first days of incubation in the non-sterile microcosms. After this period, *T. maritimum* cells could not be distinguished from the autochthonous bacterial population, because the morphology of the typical slender rod-shaped cells became spherical, about 1µm in diameter, similar to other bacteria present in the non-sterile microcosms. In the case of the sterile microcosms similar morphology changes were observed after 6 days, although a mixture of filamentous and spherical forms were detected until day 20 (Fig. 4).

After 160 days in sterile microcosms, no change in the 19 enzymatic tests assayed with API ZYM strips was observed. In addition, dot blot assays and immunoblot analysis demonstrated no differences in the serological reaction, which displayed a similar pattern as the original strain. When the RAPD technique was applied, the cluster analysis of the patterns clearly showed the same profile of the original *T. maritimum* isolate (data not shown).

Effect of selected native microbiota on the survival of T. maritimum

To determine the possible role of the native microbiota in the pronounced decrease of the survival of this bacterium in natural seawater microcosms, we conducted antagonism assays against *T. maritimum* with 12 different bacterial phenotypes isolated from these microcosms during the first two weeks of study. The identification of these bacterial isolates revealed that the *Vibrio* (6), *Pseudomonas* (3), *Alteromonas* (2) and *Aeromonas* (1) groups were dominant during this period. The antagonism assays showed that most of the bacteria tested (75 %) inhibited the growth of the *T. maritimum* strain used in this work, displaying inhibition areas ranging from 11 to 17 mm in diameter size. Only the three strains identified as *Pseudomonas* sp. were not antagonistic against *T. maritimum*. In addition, there was no evidence about the presence of specific bacteriophages for *T. maritimum* or protozoans in the natural seawater microcosms. These results seem to indicate a clear influence of the indigenous bacterial population on the rapid decrease in the recovery of the *T. maritimum* in seawater.

Resuscitation of non-culturable cells in non-sterile microcosms

To induce the resuscitation and possible growth of the non-culturable *T. maritimum* strain, the addition of fresh nutrients to the non-sterile microcosms at three different times was performed (6, 12 and 160 days). After 24 and 48 h of the addition of fresh media in each attempt of resuscitation, cells of *T. maritimum* were not recovered onto FMM supplemented with antibiotics. When the nested PCR was employed, amplification products were not obtained from these water samples.

Discussion

Although tenacibaculosis caused by *T. maritimum* is a disease affecting important marine fish, the behaviour of the organism outside the host is unknown. In fact, the route of infection and possible role of the marine environment as a reservoir of this pathogen are not yet clarified. Laboratory microcosm experiments using pure cultures have provided much of the current knowledge about microbial physiology of the pathogenic bacteria (McKay, 1992). Currently there is an extensive literature dealing with the effect of physicochemical factors on the survival of a variety of microorganisms, but the majority of studies were done only under sterile conditions. Therefore, consideration should be taken in interpreting results of laboratory studies into the field situation.

In the present study, when survival of *T. maritimum* was examined in sterile microcosms, the bacterium was able to survive in the culturable state for more than 5 months. Our results showed clearly that when at least 0.1% of the inoculated *T. maritimum* cells stayed culturable, the total cell counts determined by DAPI staining remained at very high levels (10^6 cells ml^{-1}, average value) during all the experimental period.

This long survival period contrasts with the short culturability of *T. maritimum* in natural seawater microcosms in which the pathogen remained culturable for only 5 days. The drastic decline in the culturability in non-sterilized seawater microcosms suggests an inhibitory effect of the natural aquatic microbiota on the growth and survival of this bacterium. A similar decline in culturability has been recently reported for *Aeromonas hydrophila* and *Vibrio vulnificus* in natural seawater microcosms (Maalej *et al.*, 2004; Marco-Noales *et al.*, 2004). With respect to the increase in CFU during the first days of the experiment, it can be explained by availability of natural nutrient (dissolved organic carbon) in seawater. Similar behaviour has been reported for *V. salmonicida* and *V. anguillarum* (Hoff, 1989).

On the other hand, the loss of culturability was accompanied by a significant reduction in the number of total *T. maritimum* cells, demonstrated by the nested PCR method. In fact, when this method was performed on DNA extracted from non-sterile water after 10 days, no amplification product was observed, strongly supporting that the number of *T. maritimum* cells declined to levels below the detection limit (1.6×10^2 CFU ml^{-1}) described for this protocol (Avendaño-Herrera *et al.*, 2004c). To clarify this point, we tried to resuscitate the *T. maritimum* strain after 6 and 12 days and at the end of the experiment, but this microorganism did not show capacity of resuscitation in the tested experimental conditions. Similar results have been described for a *Tenacibaculum* sp. strain by Masuda *et al.* (2004), which was recently designated as *Flexibacter echinicida* (Masuda, personal communication). Although these findings could indicate that surviving *T. maritimum* cells may exist in level below the detection limit, they are not able to be reactivated to a culturable state. One explanation is that all cells were lysed by the autochthonous microbiota, followed by rapid degradation of the DNA. In fact, analysis of the effect of the autochthonous aquatic bacteria shows, in agreement with Pazos *et al.* (1996), that the majority of these isolates inhibit growth of *T. maritimum* cells, suggesting that bacterial antagonism plays an important role in the survival of this bacterium in the marine aquatic environment. Although other biological factors, such as bacteriophages and protozoans, are known to play an important role in the survival of a variety of bacteria in aquatic systems, the presence of these bacteriovorous microorganisms was not detected in the seawater employed in this study. However, we can not rule out some influence of specific bacteriophages and protozoan on the survival of *T. maritimum* in the sea environment.

These results seem to suggest that the seawater is not an important route of transmission of *T. maritimum*. As the Latin name of the genera indicates (Te.na.ci.ba'cu.lum. L. adj. n. *tenax* holding fast; L. neut. n. *baculum* stick) (Suzuki *et al.*, 2001), perhaps this bacterium needs to be protected in sediments, attached to particle and/or animal surface, such as fish mucus, until environmental conditions are favourable for growth. Further studies are needed to support this hypothesis.

The analysis of cell surface components and phenotypic characteristics of *T. maritimum*, after 160 days in sterile microcosms, revealed that the cells maintained the same biochemical, physiological, serological and genetic characteristics of the original strain. However, these cells were reduced in size after the third or sixth day of incubation, depending on the type of water employed. Such drastic reduction in cell size may be a survival strategy to confer resistance to environmental stress, as has been observed during the starvation survival process for several bacterial species in the aquatic environment (Novitsky and Morita, 1976; Amy and Morita, 1983; Kjelleberg and Hermansson, 1984).

It is important to point out that no difference was observed in the survival dynamics for *T. maritimum* in each seawater microcosm between the independent experiments performed, indicating the behaviour of this bacterium to be reproducible.

In conclusion, results of this study suggest that the fish pathogen, *T. maritimum*, does not remain culturable after exposure to natural seawater, indicating that the autochthonous microbiota may play an important role in the survival of this fish pathogen. On the other hand, the existence of a viable but non-culturable state in *T. maritimum* remain an open question. Because of the morphological changes which *T. maritimum* displays as it becomes non-culturable, further studies should provide a clarified understanding of the survival strategies of this pathogen, which will be helpful in developing adequate preventive control programs against marine tenacibaculosis.

Experimental procedures

Bacterial strain and growth conditions

The *T. maritimum* strain used in this work, PC424.1, was isolated in Spain in 2000 from the skin of clinically infected turbot (*Scophthalmus maximus*) and maintained frozen at –70ºC in Cryo-bille tubes (AES Laboratory, France). This strain belongs to serotype "O2" and is included in one of the main clonal lineages (IIa) described for this pathogen (Avendaño-Herrera et al., 2004a and b). It was chosen as the representative of the *T. maritimum* species because all fish isolates show a high phenotypic homogeneity. For the experiment, the bacterium was cultivated on *Flexibacter maritimus* (FMM) agar (Pazos et al., 1996) and incubated at 25ºC for 72 h.

Experimental design

Survival assays were conducted in seawater (taken from Ría de Muros in Galicia, NW Spain; salinity 36‰; pH 8.0), transported to the laboratory in cold-storage containers and kept at 4ºC until use (within 24 h). Water was filtered through 0.22-µm pore-size membranes (Millipore, Madrid, Spain) and the filtrate was autoclaved at 121ºC for 15 min. In order to assess the possible effect of biotic factors on the survival of *T. maritimum*, a parallel set of non-sterile seawater microcosms (neither filtered nor autoclaved) was also prepared. The experimental assays were conducted in triplicate into 250-Erlenmeyer flasks containing 100 ml of sterile or non-sterile seawater. Inocula were prepared from cells scrapped off from FMM plates, washed in 0.85% sterile saline solution and then resuspended and diluted in sterile seawater to achieve an initial bacterial concentration in each microcosm of approximately 10^7 cells ml^{-1}, which is similar to that routinely used in this type of study (Marco-Noales *et al*., 2004). Flasks were incubated at 15ºC, which corresponds to the mean temperature scored in the NW Spain coastal water.

Sterile and non-sterile microcosm controls without the addition of the *T. maritimum* strain were run simultaneously under exactly the same conditions as described above. Two similar experiments were independently carried out to assess the reproducibility of the results.

Direct plate counts

To determine the evolution of culturable cells in each microcosm, samples of 0.5 ml were collected aseptically, daily during the first week and then weekly. Samples were serial-diluted in 0.85% sterile saline solution and 0.1 ml of each dilution was plated on Marine agar (MA) (Pronadisa, Madrid, Spain) for the count of total heterotrophic marine bacteria. All samples were inoculated in parallel onto FMM, the most effective medium for recovery of *T. maritimum*, yielding better growth compared to heterotrophic halophilic bacteria, e.g. *Vibrio*, *Pseudomonas* and *Alteromonas* species (Pazos *et al.*, 1996). In addition, it is important to note that in the FMM agar the *T. maritimum* colonies exhibit typical features for this species: flat, pale-yellow colonies with uneven edges and strongly adherent to the medium, being an appropriate medium to distinguish this microorganism from others present of the sample (Pazos *et al.*, 1996; Avendaño-Herrera *et al.*, 2005).

Once the plate counts were negative for *T. maritimum*, samples of 1 ml were taken directly from the microcosms at later time points and seeded onto FMM. If culturable *T. maritimum* could not be recovered under these conditions, in one of the assay replicates the remaining volume of the microcosm seawater was centrifuged and the pellet resuspended in 1.5 ml in the same type of seawater to be seeded onto FMM. The MA and FMM plates were incubated at 25ºC for one week and then selective counts of colony forming units (CFU) were carried out to determine the presence of the inoculated *T. maritimum* isolate in each microcosm. All plating was done in duplicate and the number of viable bacterial cells in the microcosms were calculated from the dilution that gave between 30 and 300 colonies on the agar plates. The bacterial numbers are indicated as an average of the bacterial numbers in the three parallel microcosms ± standard deviation.

Total cell counting by epifluorescent microscopy

The number of total bacteria in sterile and non-sterile microcosms was determined by epifluorescence microscopy, employing 4',6-diamidino-2-phenilindone staining (DAPI, Sigma Aldrich Química, S.A., Madrid, Spain) (Porter and Feig, 1980). Samples of 1 ml, taken daily during the first week and then weekly, were fixed with formalin, diluted and stained with DAPI (final concentration 0.1 µg ml^{-1}). After 10 min incubation, samples were filtered onto 0.22-µm black Nucleopore filters (GTBP filtertype, Millipore), which were previously washed twice with 2 ml of sterile saline solution to avoid the lysis of bacteria on the filters. The prepared filters were observed using epifluorescence microscopy (Olympus BX50) on at least 10 fields randomly selected for a total count of more than 400 cells, and the numbers of bacteria were calculated.

Single-round and nested PCR analysis

One ml samples from each microcosm were taken daily during the first week and then weekly and placed in 1.5 ml sterile tubes, which were then centrifuged at 12000X *g* for 5 min. Extraction of DNA from bacterial pellets was performed using InstaGene Matrix (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions. After the extraction, each DNA sample was maintained at –20ºC until they were used for PCR reactions.

PCR analysis was used for the detection of *T. maritimum* in all microcosms using primers described by Toyama *et al*. (1996). To increase the sensitivity of the single-round PCR assay, a nested PCR was conducted as described by Avendaño-Herrera *et al.* (2004c). Ready-To-Go^{TM} PCR beads (Amersham Pharmacia Biotech, Barcelona, Spain) were used for the reaction, and this was carried out according to the parameters described to each published PCR protocol. A positive result was indicated by the presence of an amplification product of 1088 bp after electrophoresis through a 1% (w/v) agarose gel. All analyses were carried out with DNAs obtained in two different extractions from each microcosm.

Analysis of cellular changes and surface components

To determine if *T. maritimum* suffered cellular changes during the experimental period, samples of sterile and non-sterile microcosms were periodically visualized by light microscopy, as well as by epifluorescence microscopy observing the filters with DAPI dye. Moreover, the possible changes in phenotypic and genetic characteristics of *T. maritimum* were evaluated using the culturable cells grown 160 days after inoculation in sterile microcosms. Biochemical alterations during the starvation period were examined using the miniaturized API ZYM system (bioMérieux, Madrid, Spain) according to the manufacturer's instructions, with the exception of the incubation temperature that was fixed at 25ºC (Avendaño-Herrera *et al.* 2004a). Serological changes of the *T. maritimum* isolate were analyzed by dot-blot using the respective O-antigen and the antiserum against PC424.1 raised in New Zealand rabbits. To determine antigenic alterations in the component of the bacterial cell wall, we analyzed the lipopolysaccharide (LPS) profile by Western blot analysis, according to Avendaño-Herrera *et al*. (2004a). RAPD method was also employed to analyse the genetic pattern of the starved *T. maritimum* strain, which was compared with the original isolate using the method described by Avendaño-Herrera *et al*. (2004b).

Resuscitation conditions

After 5 days from the beginning of the experiment the plate counts were confirmed to be negative for *T. maritimum* only in the non-sterile microcosms and the reversion to a culturable state was attempted by addition of a sterile solution of casamino acids (20%) and glucose (50%) 1: 1000 (v/v) to each of the non-sterile microcosms, according to previous studies (data not shown). The resuscitation experiments were also conducted after 12 days and at the end of the experiment (day 160). Flasks were incubated at 25ºC for 48 h. As *T. maritimum* PC424.1 strain proved to be resistant to the oxolinic acid and the vibriostatic agent O/129 (Baxa *et al*., 1988; Avendaño-Herrera *et al*., 2005), 1 ml samples taken directly after 24 and 48 h from the non-sterile microcosms were plated on FMM containing oxolinic acid (40 μg ml^{-1}, Sigma) and the vibriostatic agent O/129 (30 μg ml^{-1}, Sigma) to inhibit the growth of the autochthonous microorganisms. CFU were enumerated after 72 h of incubation at 25ºC to confirm the absence or presence of *T. maritimum* cells. Simultaneously, 1 ml samples from the non-sterile microcosms were collected at the same periods after supplementation, to extract genomic DNA in order to process single-round and nested PCR amplification as described above.

Screening of the autochthonous aquatic microbiota for inhibitory activity

To determine the possible role of the native bacterial population in the survival of *T. maritimum*, representatives of the numerically most abundant morphotypes grown on MA plates from samples of non-sterile microcosms seeded with *T. maritimum* were chosen for isolation. The pure bacterial cultures obtained were identified at genus level according to general procedures of Bergey's Manual of Systematic Bacteriology (Hansen and Sørheim, 1991; Holt *et al.*, 1994) and maintained frozen at –70ºC in Cryo-bille tubes.

All isolates were screened for production of inhibitory substances on growth of *T. maritimum* PC424.1, according to the procedure described by Lemos *et al.* (1985). The bacteria were cultured on MA plates and a small amount of cell paste was scraped off and deposited onto the surface of FMM seeded with the *T. maritimum* strain. After a diffusion time of 30 min, plates were incubated at 25ºC for 72 h. A clear zone of inhibition of *T. maritimum* around the cell paste indicated antibacterial activity.

Moreover, the possible presence of bacteriophages and protozoans in the natural seawater employed in this study was also examined. Bacteriophages were evaluated by the double-agar-layer technique using an enrichment procedure in which seawater samples were amended with an equal volume of FMM broth containing a 1:100 (vol/vol) dilution of an overnight culture of *T. maritimum*. Enrichment cultures were incubated overnight and centrifuged to eliminate all viable bacterial cells. Bacteriophages were detected by a plaque assay with lawns of *T. maritimum* strain. In the case of protozoans, 100 ml samples were centrifuged, resuspended in 1 ml of the same seawater and filtered to be used for microscopy observation.

Acknowledgements

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Figure legend

Fig. 1. Survival patterns of the *T. maritimum* strain PC424.1 in microcosms containing sterilized seawater. (A) first week. (B) the entire experimental period. Microcosms were assayed by DAPI direct counts (●) and plate counts on FMM agar medium (□). Bars indicate standard deviation.

Fig. 2. Survival pattern of the *T. maritimum* strain PC424.1 in the presence of autochthonous microbiota in non-sterile microcosms at 15ºC. (A) Behaviour during the first week. (B) Behaviour during the entire experimental period. Culturability was determined on FMM agar plates for *T. maritimum* (□) and on Marine agar for autochthonous bacteria (▲). Cell numbers quantified by DAPI direct counts (●). Arrowheads indicate the addition of fresh medium to the microcosms. Arrows indicate that *T. maritimum* CFU values are below the detection limit. Bars indicate standard deviation.

Fig. 3. Detection of *T. maritimum* in non-sterile microcosms kept for 11 days under survival conditions by single round PCR (A) and nested PCR (B). Lanes: M, AmpliSize Molecular Ruler (50-2000-bp ladder, Sigma); Dw, negative control (DNA extracted non inoculated sterile microcosms); Ct, positive control (DNA extracted from pure culture of the original strain); 1 to 11, DNA extracted from the samples taken at days 1, 3, 4, 5, 7, 8, 9, 10 and 11 respectively. Numbers on the left indicate the position of molecular size marker in bp. Numbers on the right indicate the size of the specific amplified products in bp.

Fig. 4. Fluorescence microscopy of a sample of *T. maritimum* population collected from a sterile microcosm after 10 days of incubation at 15ºC. The image shows changes in the morphology and size of *T. maritimum*. Arrow indicates cells that did not change their morphology from rod to cocci. Bar length, 20 µm.

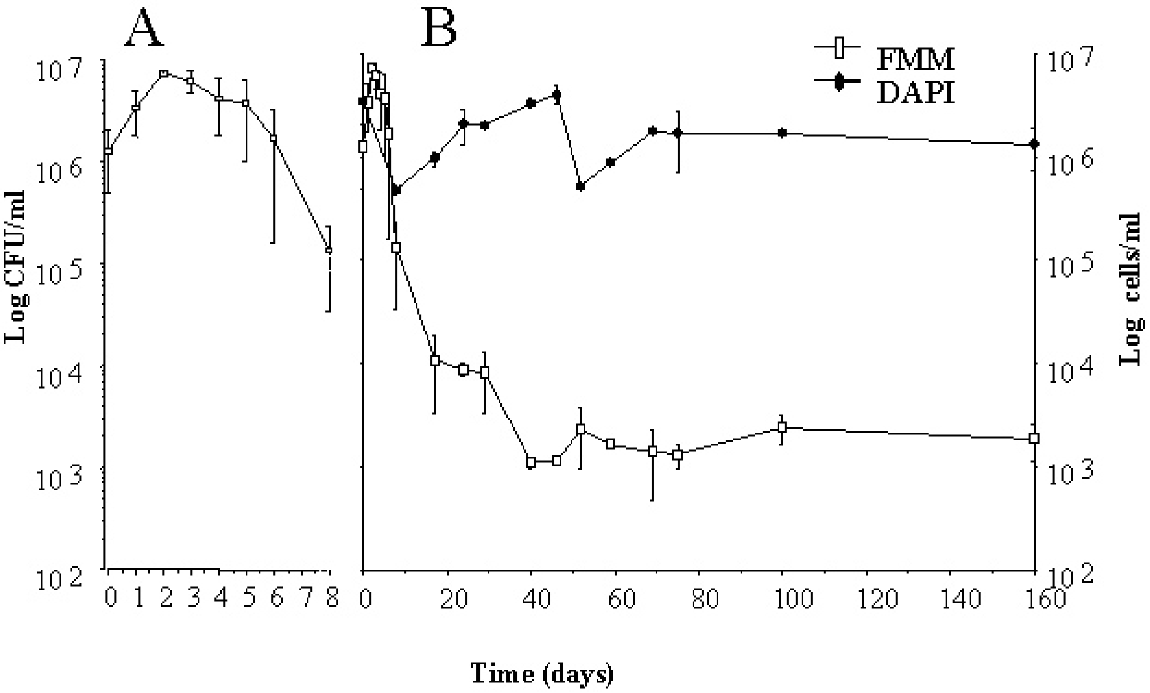


Fig. 1. Avendaño-Herrera et al.

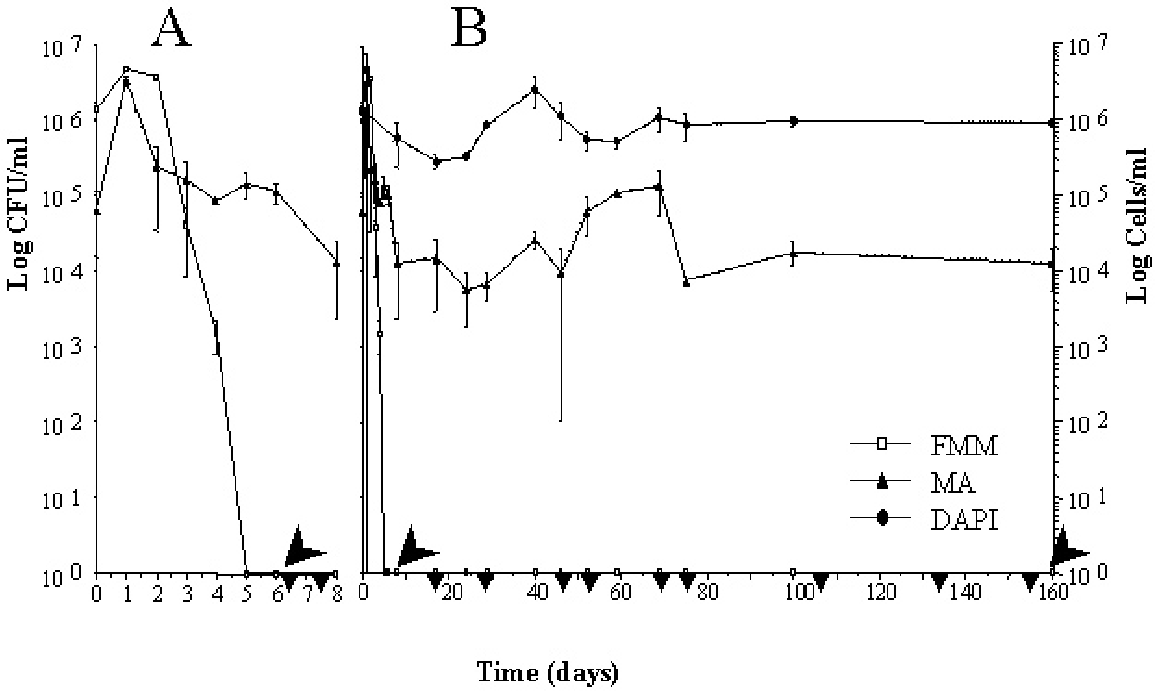


Fig. 2. Avendaño-Herrera et al.

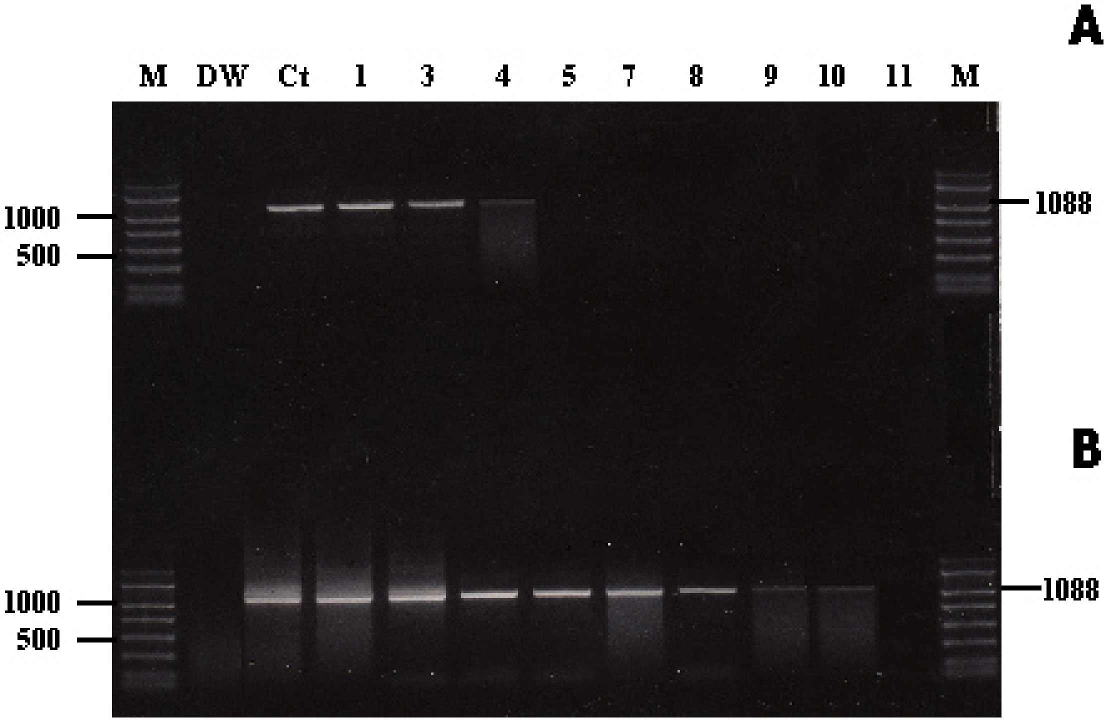


Fig. 3. Avendaño-Herrera et al.

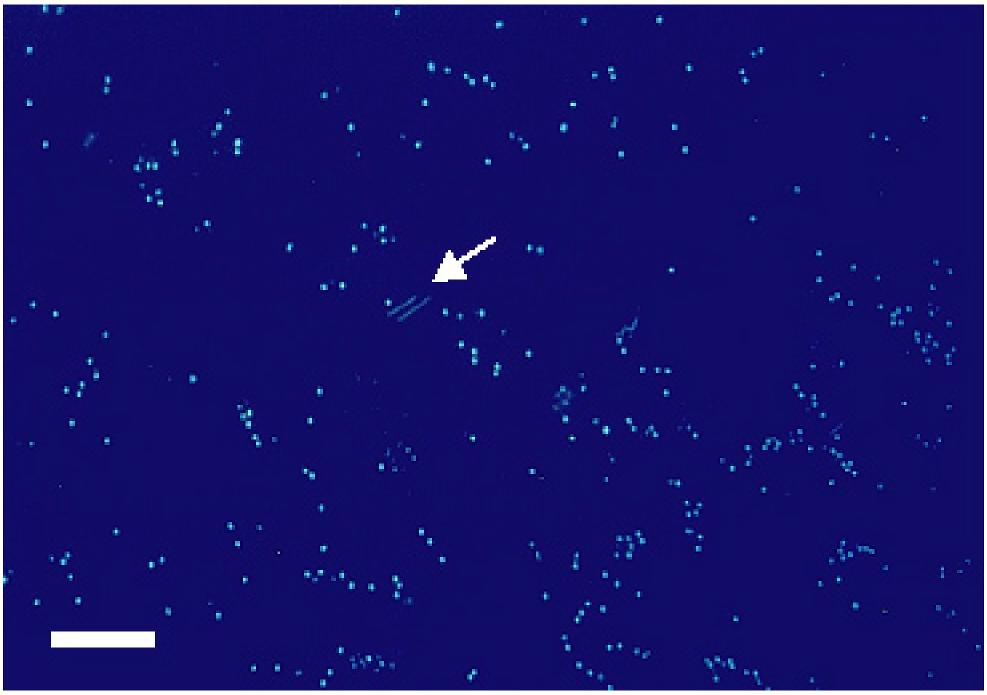


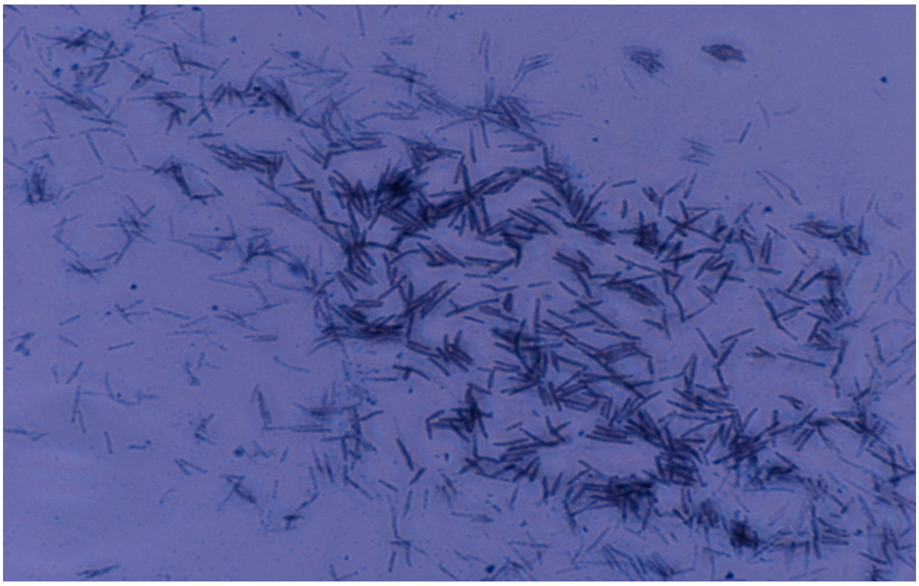
Fig. 4. Avendaño-Herrera et al.

VI.B. LÁMINAS COMPLEMENTARIAS A LAS PUBLICACIONES

Lámina 1. Morfología celular de la cepa PC424.1 al inicio de los experimentos de supervivencia en agua de mar artificial, las cuales fueron observadas mediante microscopia y usando tinción de cristal violeta.

Lámina 2. Morfología de *T. maritimum* durante los experimentos de supervivencia en los microcosmos de agua de mar natural observada mediante microscopía de epifluorescencia utilizando DAPI. Se observan células que mantienen su forma filamentosa después de 2 días de incubación a 15ºC, las cuales están rodeadas por células de *T. maritimum* que han sufrido cambios de morfología y tamaño, así como otras bacterias presentes en el agua de mar. La barra representa 20 µm.

Lámina 1.



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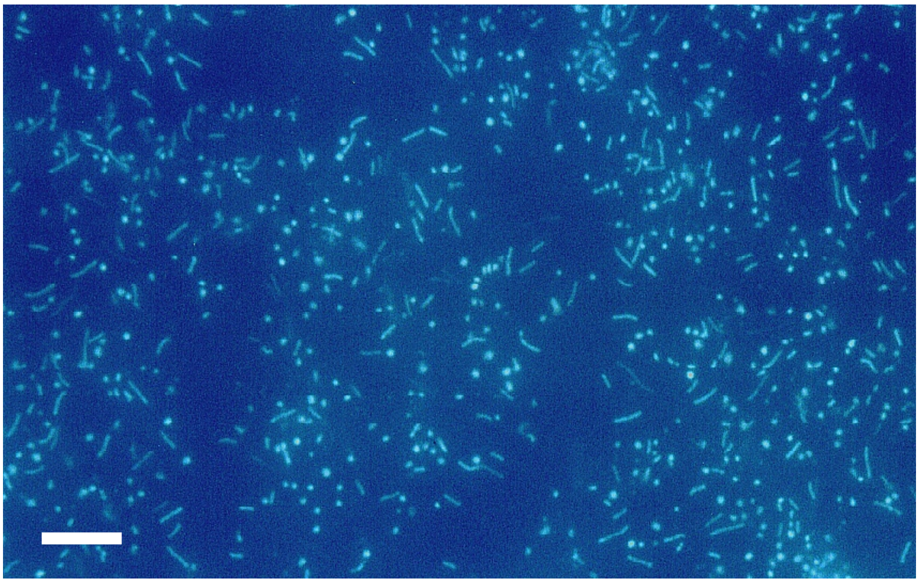

VII. FACTORES DE VIRULENCIA

VII.A. PUBLICACIONES

Artículo nº 7: Iron uptake mechanisms in the fish pathogen *Tenacibaculum maritimum*. **Applied and Environmental Microbiology (2005). 71**: 6947-6953.

CONTENIDOS:

- Crecimiento en condiciones limitantes de hierro.
- Producción de sideróforos y bioensayos.
- Utilización de hemina, hemoglobina, transferrina y citrato férrico amónico.
- Evaluación indirecta de la existencia de posibles receptores de membrana para componentes de tipo hemo como hemina y rojo Congo.
- Actividades enzimáticas presentes en los aislados de *Tenacibaculum maritimum* crecidos en condiciones de limitación de hierro.
- Inducción de proteínas en condiciones de limitación de hierro.

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Iron Uptake Mechanisms in the Fish Pathogen *Tenacibaculum maritimum*

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We present here the first evidence of the presence of iron uptake mechanisms in the bacterial fish pathogen *Tenacibaculum maritimum*. Representative strains of this species, with different serotypes and origins, were examined. All of them were able to grow in the presence of the chelating agent ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDHA) and also produced siderophores. Cross-feeding assays suggest that the siderophores produced are closely related. In addition, all *T. maritimum* strains utilized transferrin, hemin, hemoglobin, and ferric ammonic citrate as iron sources when added to iron-deficient media. Whole cells of all *T. maritimum* strains, grown under iron-supplemented or iron-restricted conditions, were able to bind hemin, indicating the existence of constitutive binding components located at the *T. maritimum* cell surface. This was confirmed by the observation that isolated total and outer membrane proteins from all of the strains, regardless of the iron levels of the media, were able to bind hemin, with the outer membranes showing the strongest binding. proteinase K treatment of whole cells did not affect the hemin binding, indicating that, in addition to proteins, some protease-resistant components could also bind hemin. At least three outer membrane proteins were induced in iron-limiting conditions, and all strains, regardless of their serotype, showed a similar pattern of induced proteins. The results of the present study suggest that *T. maritimum* possesses at least two different systems of iron acquisition: one involving the synthesis of siderophores and another that allows the utilization of heme groups as iron sources by direct binding.

Tenacibaculum maritimum (formerly *Flexibacter maritimus*) (48) is the etiological agent of an ulcerative disease known as marine flexibacteriosis or tenacibaculosis, which affects a large number of marine fish in the world and thus has considerable economic significance to aquaculture producers. The infection has diverse clinical manifestations depending on the species and age of fish, the most significant symptom being the presence of gross lesions on the body surface (11, 16).

Although the phenotypic, antigenic, and molecular characteristics of *T. maritimum* have been examined by several authors (for a review, see reference 49), the actual factors determining the virulence of this pathogen have not yet been elucidated. Some synergistic interactions of the toxins contained in extracellular products and a hemolysin might be involved in *T. maritimum* infections (8). Moreover, pathological properties of the bacterium, such as a strong adherence to the skin mucus of different fish species and the capacity to resist its bactericidal activity (31), have been pointed out as possible virulence factors.

It is well known that the ability to take up iron during infection is an essential factor in the pathogenicity of several bacteria, being necessary for the pathogen multiplication. However, the levels of free iron in the biological fluids within the animal host is often very limited because the element is strongly bound to a high-affinity iron-binding proteins. To obtain this unavailable iron, most pathogenic bacteria have developed iron uptake systems that usually involve two components: (i) low-molecular-weight siderophores released by the bacteria that will chelate iron and subsequently transfer it to the pathogen and (ii) iron-regulated outer membrane proteins (IROMPs) that function as receptors of the iron-siderophores complexes (36, 43). These mechanisms of iron acquisition have been linked to the virulence of different fish pathogens such as *Vibrio anguillarum* (*Listonella anguillarum*) (35, 53), *Aeromonas salmonicida* (21, 25), *Photobacterium damselae* subsp. *piscicida* (17, 30), and *Edwardsiella tarda* (23, 24). However, other pathogenic bacteria have developed mechanisms to acquire iron relying on the interaction between specific microbial receptors and host transferrin or heme-containing compounds (28, 39, 51).

Recently, in vitro studies have shown that iron seems to be an important factor for the resuscitation of a *Tenacibaculum* sp. strain from the viable but nonculturable state (32), suggesting that, as in many bacteria, this micronutrient plays a key role in the growth of *T. maritimum*. Therefore, we sought to get a first insight into the mechanisms that *T. maritimum* possess for iron assimilation from the host tissues.

MATERIALS AND METHODS

Bacterial strains. A total of 17 *T. maritimum* strains were included in the present study (Table 1). This collection comprises 15 strains isolated from six different marine fish species, which belongs to the different serotypes and clonal lineages described within this pathogen (4, 5, 6), and two reference strains (NCIMB 2154^T and 2158) from the National Collection of Industrial and Marine Bacteria (Aberdeen, United Kingdom). These strains were confirmed as *T. maritimum* by PCR-based analysis as described by Toyama et al. (50). For all experiments, *T. maritimum* cells were routinely cultivated on *Flexibacter maritimus* medium (FMM) agar or broth at 25°C for 48 h. FMM (0.5% peptone [Difco Laboratories, Madrid, Spain], 0.05% yeast extract [Oxoid, Ltd., Basingstoke, Hampshire, England], and 0.001% sodium acetate [Sigma Aldrich

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TABLE 1. *T. maritimum* strains used in this study and results of growth under iron-limiting conditions and siderophore production

Strain	Isolation source	Origin	Yr of isolation	Serotype[a]	Genetic group[a]	MIC (μM) EDDHA	Chemical test[b]	
							CAS agar	CAS in liquid
LR2P	Sole (*Solea solea*)	Spain	1995	O1	I	130	1.38	−0.18
PC503.1	Sole (*Solea senegalensis*)	Spain	2001	O1	I	130	1.36	−0.15
PC528.1	Sole (*Solea senegalensis*)	Spain	2002	O1	I	160	1.72	−0.31
AZ203.1	Sole (*Solea senegalensis*)	Spain	2001	O1	I	140	1.33	−0.18
ACC6.1	Sole (*Solea senegalensis*)	Portugal	2003	O3	II	180	2.0	−0.48
LgH35-O3[c]	Sole (*Solea senegalensis*)	Spain	2003	O3	ND	140	1.38	−0.23
PC424.1	Turbot (*Scophthalmus maximus*)	Spain	2000	O2	II	160	1.44	−0.47
PC473.1	Turbot (*Scophthalmus maximus*)	Spain	2001	O2	II	160	1.83	−0.23
ACR104.1	Turbot (*Scophthalmus maximus*)	Spain	2001	O2	II	150	1.61	−0.14
JIP 24/99[d]	Turbot (*Scophthalmus maximus*)	Spain	1999	O2	ND	150	1.47	−0.17
PC538.1	Gilthead seabream (*Sparus aurata*)	Spain	2002	O1	I	170	1.96	−0.30
DOB102	Gilthead seabream (*Sparus aurata*)	Spain	2002	O1	I	160	1.44	−0.14
JIP 32/99[d]	Sea bass (*Dicentrarchus labrax*)	France	1999	O1	II	140	1.38	−0.23
LVDH 1577.01[d]	Sea bass (*Dicentrarchus labrax*)	France	2003	O3	ND	150	1.42	−0.14
DBA4a[d]	*Seriola quinqueradiata*	Japan	1986	ND[e]	II	130	1.47	−0.15
NCIMB 2158	Sole (*Solea solea*)	United Kingdom	1981	O2	III	170	1.67	−0.23
NCIMB 2154^{T}	Japanese seabream (*Pagrus major*)	Japan	1977	O2	III	180	2.17	−0.50

[a] Data from Avendaño-Herrera et al. (4, 5, 6).

[b] The CAS test was performed by using the chemical assay of Schwyn and Neilands (50) in solid and liquid media. Siderophore production on CAS plates was expressed as the ratio of diameter of orange halo to the diameter of growth. CAS values in liquid medium are expressed as the absorbance values at 630 nm of supernatants (a positive reaction gives a decrease in A_{630} of CAS dye). The results for the Arnow and Csaky assays were negative.

[c] Supplied by M. A. Moriñigo, Department of Microbiology, University of Malaga, Malaga, Spain.

[d] Supplied by J. F. Bernardet, Unité de Virologie et Inmunologie Moleculaires, INRA, Paris, France.

[e] ND, not determined.

Química, S.A., Madrid, Spain] supplemented with 1.5% agar [Cultimed Panreac Química S.A., Barcelona, Spain]; pH 7.2 to 7.4) was prepared with seawater as diluent according to the original descriptions (41). Stock cultures were maintained frozen at −80°C in Cryo-bille tubes (AES Laboratories, France).

Growth under iron-limiting conditions. The ability of the *T. maritimum* strains to grow in the presence of the nonassimilable iron chelator ethylenediamine-di-(*o*-hydroxyphenyl-acetic acid) (EDDHA; Sigma) was determined by using FMM broth as a basal medium due to the known strict halophilic nature of this bacterium (7). MICs were obtained by adding increasing concentrations of EDDHA and were defined as the lowest concentration at which no bacterial growth was observed. All experiments were carried out in triplicate with three different batches of media.

Production of siderophores. Production of compounds with siderophore activity was tested by the universal chemical assays of Schwyn and Neilands (45) in solid and liquid media. The assays were performed by spotting 10 μl of each bacterial culture grown in iron-limiting conditions onto a modified chromoazurol S (CAS; Sigma) agar, which was prepared with FMM as the basal medium. The siderophore levels produced by the strains on plates were expressed as the ratio of orange halo diameter to growth diameter after 72 h of incubation. For siderophore detection in liquid media, supernatants from culture grown in FMM broth containing 80 μM EDDHA were mixed with CAS supernatant solution, and the absorbance of mixture was measured at 630 nm. As a positive control we used a *V. anguillarum* wild-type strain 775, and as a negative control we used a non-siderophore-producing strain *V. anguillarum* 775::Tn*1-5* (30).

The presence of phenolic compounds and/or hydroxamic acids were detected in cell-free supernatants obtained from iron-depleted cultures by the colorimetric assays of Arnow (3) and Csáky (14) as modified by Andrus et al. (1), respectively. The positive controls for phenolate-type siderophores consisted in the iron-limiting FMM broth containing 10 μM 2,3-dihydroxybenzoic acid, while the positive control for hydroxamate-type siderophores was the same media with 10 μM purified aerobactin.

Cross-feeding assays. To test the ability of each *T. maritimum* strain to induce the growth of other strains of the same species subjected to iron starvation, cross-feeding assays were used. Briefly, *T. maritimum* strains were grown in FMM broth in iron-limiting and iron-replete conditions (80 μM EDDHA and 20 μM $FeCl_3$, respectively) and centrifuged at 12,000 × *g* for 5 min. Cell-free supernatants from the strains to be tested for the production of siderophore-like compounds were pipetted onto sterile filter paper disks and, after being dried, were placed on the agar surface of FMM plates seeded with the strain to be used as indicator and containing a concentration of EDDHA higher than the MIC for that strain. Growth around the disks indicates the production by the tested strain of a diffusible siderophore that can be utilized by the indicator strain. As a negative control a non-siderophore-producing strain *V. anguillarum* 775::Tn*1-5* was used (30).

Growth with different iron sources. FMM broth supplemented with EDDHA at a concentration sufficient to achieve total growth inhibition of the strains tested was supplemented by the utilization of various iron sources: transferrin (human), apotransferrin (human), hemin (bovine), hemoglobin (bovine), and ferric ammonium citrate (Panreac Química S.A.). Transferrin and apotransferrin (Sigma) were dissolved to 1 mM in 100 mM Tris, 150 mM NaCl, and 50 mM $NaHCO_3$ (pH 8.0). Hemin (Sigma) stock solution was prepared in 10 mM NaOH and hemoglobin (Sigma) in distilled water. All stock solutions were sterilized by filtration through 0.22-μm-pore-size membrane (Millipore) and maintained at −20°C until use.

Iron-containing compounds were used at concentrations ranging between 0.1 and 20 μM. FMM broth plus EDDHA tubes supplemented with iron components were seeded with a 1:100 (vol/vol) inoculum from an overnight culture in FMM broth and were incubated at 25°C. The absorbance of the mixture was determined after 48 and, if necessary, 72 h of incubation at 600 nm. Growth with heme compounds as the only iron source was also examined on FMM solid medium plus EDDHA by a standard radial diffusion method. Sterile paper disks impregnated with 10 μl of either hemin or hemoglobin at a concentration of 0.1, 5, 10, and 20 μM were placed onto the previously inoculated medium. The plates were observed after 72 h of incubation at 25°C to detect halos of growth around disks. Moreover, in order to evaluate the effect of hemin and hemoglobin on growth rates, bacterial growth curves were obtained. Inocula obtained as described above were added to iron-restricted medium containing these iron sources at a concentration of 5 μM and were incubated at 25°C. Samples were taken every 6 h and the optical density at 600 nm was measured.

Hemin-binding assays. The existence of possible membrane receptors for heme compounds was determined by Congo red and hemin-binding assays in liquid and solid-phase according to the procedures described by Kay et al. (26) and Mazoy and Lemos (34). *T. maritimum* cells grown in FMM containing 20 μM $FeCl_3$ and FMM plus 80 μM EDDHA were harvested by centrifugation, washed in sterile 0.9% saline solution, and suspended in saline solution (20 ml) to an optical density of 1.5 at 620 nm. Congo red (Sigma) or hemin was added to a final concentration of 30 or 40 μg ml^{-1}, respectively. One-milliliter samples were immediately removed and centrifuged at 13,000 × *g* for 30 s, and the supernatant was assayed spectrophotometrically for Congo red (A_{488}) or hemin (A_{400}). The remaining cells were shaken at 25°C and assayed at 30-min intervals for residual

TABLE 2. Results of cross-feeding experiments in *T. maritimum* grown in FMM broth under iron-replete and iron-restricted conditions

Strain	Ability to cross-feed							
	Producer strains grown in iron-limiting conditions (FMM + 80 μM EDDHA)				Producer strains grown in iron-replete conditions (FMM + 20 μM $FeCl_3$)			
	PC503.1	PC424.1	ACC6.1	*V. anguillarum*[a] 775::Tn*1-5*	PC503.1	PC424.1	ACC6.1	*V. anguillarum* 775::Tn*1-5*
T. maritimum								
PC503.1	+	+	+	−	−	−	−	−
PC424.1	+	+	+	−	−	−	−	−
ACC6.1	+	+	+	−	−	−	−	−
Control								
V. anguillarum 775::Tn*1-5*	−	−	−	−	−	−	−	−

[a] Strain siderophore deficient, receptor proficient. Donated by J. H. Crosa, Oregon Health and Science University.

Congo red or hemin in the supernatant as described above. The experiment was performed in duplicate.

For the whole-cell binding assay in solid-phase, bacterial strains were cultured under iron-limited and iron-supplemented conditions and collected by centrifugation at 4,000 × *g* for 5 min. These cells were washed in saline solution and resuspended to an optical density of 0.8 at 580 nm. A 30-μl volume of the cell suspension was placed onto nitrocellulose membranes (0.45-μm pore size) in a dot blot manifold (Schleicher & Schuell, Inc., Dassel, Germany). After immobilization the membranes were air dried, blocked with 2% gelatin in Tris-buffered saline (TBS; 50 mM Tris-HCl supplemented with 0.9% NaCl [pH 8.0]), incubated for 2 h with hemin (10 μM in TBS), immersed for 30 min in 12.5% trichloroacetic acid, washed in distilled water, and stained with 3,3′-dimethoxybenzidine (DMB; Sigma). The DMB solution (50 mg of DMB in 15 ml of distilled water) was freshly prepared just before use and stirred for 15 min, and 5 ml of a 0.5 M sodium citrate buffer (pH 4.4) and 100 μl of 30% H_2O_2 were added. After staining for 3 to 5 min, the membrane was washed with water to clear the background. To determine whether hemin binding was protein associated, bacterial suspensions were incubated with proteinase K solution (500 μg ml^{-1}) in phosphate-buffered saline (pH 7.4) for 1 h at 37°C before the solid-binding assays. *Escherichia coli* HB101 and *V. anguillarum* strain 775 were used as negative and positive controls, respectively (17).

Analysis of enzymatic activities. The enzymatic activities of *T. maritimum* grown in iron-rich and iron-restricted media were comparatively evaluated in order to determine the possible role of iron in the enzymatic expression. The hydrolysis of the following substrates were examined by using FMM as basal medium supplemented with 80 μM EDDHA or 20 μM $FeCl_3$: 2% (wt/vol) gelatin (Sigma), 1% (wt/vol) starch (Merck, Darmstadt, Germany), 3% (wt/vol) carboxymethyl cellulose (Sigma) and 0.1% (wt/vol) esculin (Merck). These substrates are normally used for the biochemical characterization of *T. maritimum* (4). All of these activities were determined by standard radial diffusion method with filter paper disks impregnated with 10 μl of each live *T. maritimum* cells and with bacterial-cell-free supernatants. All tests were read after 72 h at 25°C. In addition, the presence and levels of enzymatic activity were examined with the API ZYM (bioMèrieux) miniaturized system of both live cells and supernatant of each *T. maritimum* strain grown in iron-supplemented and iron-deficient conditions according to the manufacturer's instructions with the exception of the incubation temperature that was fixed at 25°C.

Analysis of membrane proteins. The presence of iron-regulated proteins was tested by growing each *T. maritimum* strain in FMM broth containing 80 μM EDDHA and by comparing the patterns with those appearing when the strains were grown in FMM liquid plus 20 μM $FeCl_{3.}$ Total and outer membrane proteins were obtained as previously described Avendaño-Herrera et al. (4) and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27) with 12% (wt/vol) acrylamide in the resolving gel and 4% (wt/vol) acrylamide in the stacking gel using a Mini Protean 3 cell apparatus (Bio-Rad). Both total membrane and outer membrane proteins were adjusted to a concentration of 40 ± 2 μg with bovine serum albumin as the standard according to the methods described by Bradford (10). After electrophoresis (60V for 90 min), the proteins were stained with 0.05% (wt/vol) Coomassie blue R (Sigma) in 25% (vol/vol) propan-2-ol-10% (vol/vol) acetic acid. Gels were destained with 10% acetic acid and 40% methanol and photographed. All experiments were carried out with proteins obtained in two different extractions for each bacterial strain.

Hemin-binding by bacterial membrane protein extracts. Binding of hemin by membrane protein extracts was assayed as previously described (17). Briefly, 20 μl of either total or outer membrane proteins containing 80 or 40 μg of protein in distilled water were immobilized on nitrocellulose membranes, which were then blocked with gelatin, incubated with hemin, immersed in 12.5% trichloroacetic acid, washed in distilled water, and stained with DMB, as described above. The effect of protease treatment on hemin binding by protein extracts was evaluated by incubating bacterial total membrane and outer membrane protein with proteinase K solution (500 μg ml^{-1}) in phosphate-buffered saline for 1 h at 37°C. The samples were then loaded onto nitrocellulose membrane and subjected to a dot blot assay to evaluate the binding of hemin.

RESULTS AND DISCUSSION

The presence of efficient iron uptake systems has been considered an important virulence determinant for several patho-

TABLE 3. Growth of the three *T. maritimum* strains with different iron sources in FMM broth supplemented with EDDHA at a concentration sufficient to achieve total growth inhibition of the strains tested

Iron source	Growth of *T. maritimum* isolates[a]														
	PC503.1 at concn (μM)					PC424.1 at concn (μM)					ACC6.1 at concn (μM)				
	0.1	1	5	10	20	0.1	1	5	10	20	0.1	1	5	10	20
Hemin	++	++	++	++	++	++	++	++	++	++	++	++	++	+++	+++
Hemoglobin	++	++	++	++	++	++	++	++	++	++	+++	+++	+++	+++	++
Transferrin	++	+	+	−	−	++	+	+	−	−	++	+	+	−	−
Apotransferrin	++	+	+	−	−	++	+	+	−	−	++	+	+	−	−
Ferric ammonium citrate	++	++	+++	++++	++++	++	++	+++	+++	+++	++	++	+++	+++	+++

[a] Growth with different iron sources expressed as absorbance values measured at 600 nm after 72 h of incubation: −, <0.040; +, 0.040 to 0.080; ++, 0.080 to 0.150; +++, >0.150.

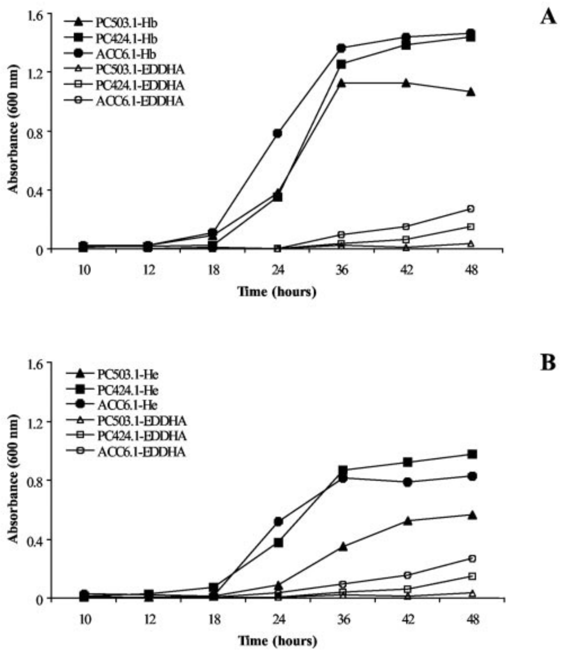

FIG. 1. Utilization of hemoglobin (Hb) at 5 μM (A) and hemin (He) at 5 μM (B) as iron sources by *T. maritimum* strains grown in FMM medium containing EDDHA.

gens (40). These microorganisms must compete with the host for iron, and in many instances do so via the production of high-affinity iron chelators which, in conjunction with cell surface receptors, transport iron into cells (19, 43). Although tenacibaculosis caused by *T. maritimum* is a disease affecting important marine fish (see reference 49 for a review), the studies about its virulence mechanisms are scarce (8). For this reason, in the present study we studied the presence of iron acquisition mechanisms in *T. maritimum* which could have an important role in the pathogenicity for fish.

Growth in iron-limiting conditions and siderophore production. The results obtained showed that the 17 *T. maritimum* isolates tested were able to grow under iron-limiting conditions with MICs for the iron-chelating agent EDDHA ranging from 130 to 180 μM (Table 1). In fact, the siderophore production assays revealed that all *T. maritimum* strains gave positive reaction on CAS agar plates and CAS liquid test, showing a good correspondence between both assays. The *T. maritimum* isolates showed halo/growth diameter ratios ranging from 1.33 to 2.17 and absorbance values ranging from −0.14 to −0.50 (Table 1). However, although all strains produced siderophores, the chemical assays performed with cell-free supernatants showed that they do not contain typical hydroxamate- or phenolate-type compounds. The occurrence of a siderophore which is neither a phenolate nor a hydroxamate has been reported for other bacteria (22, 47). Further studies are needed to elucidate the chemical structure of the siderophore produced by *T. maritimum*.

Based on the biochemical homogeneity of the bacterium and the results of siderophore production assays, three *T. maritimum* strains isolated from sole (PC503.1 and ACC6.1) and turbot (PC424.1), representing the main serotypes described in this pathogen (4, 6), were selected to be used in the subsequent studies.

The cross-feeding assays showed that the three strains tested were able to produce iron-sequestering compounds only when they were cultured under iron-limiting conditions (Table 2). Each strain was able to use the compounds secreted by itself, as well as the compounds secreted by the other two strains. This suggests that all *T. maritimum* strains studied likely produce highly related siderophores.

Growth with different iron sources. Another finding in our study was that when high inhibitory concentration of EDDHA in the culture medium was imposed, all *T. maritimum* isolates utilized transferrin, apotransferrin, hemin, hemoglobin, and ferric ammonium citrate as the only iron source. As observed in Table 3, all compounds could stimulate growth in all strains at the lowest concentration tested (0.1 μM), with an optimum concentration of 10 μM for hemin and hemoglobin. It is interesting to denote that, with the exception of ferric ammonium citrate, higher concentration of the compounds tested did not result in an increase of cells yield, being more evident when the assays with transferrin and apotransferrin were performed. Similar behavior has been reported for *V. anguillarum* (33). When the growth kinetics of all *T. maritimum* strains under hemin or hemoglobin-conditions were tested, strains ACC6.1 and PC424.1 exhibited very fast growth (Fig. 1). According to these observations, *T. maritimum* can efficiently use iron contained in hemin or hemoglobin in agreement with the observations reported for other pathogenic microorganisms (for a review, see reference 38).

Hemin-binding assays. To determine whether the *T. maritimum* species contain iron-regulated membrane binding involved in iron acquisition from heme compounds, we grew all strains in the presence of 80 μM EDDHA and measured the Congo red-binding ability of these cells. The three *T. maritimum* strains did not show an increase in Congo red absorption in relation to the cells grown in FMM without EDDHA. The same effect was noted when the hemin-binding ability of *T. maritimum* was measured (Fig. 2). Previous reports have shown that some pathogenic bacteria can absorb hemin and the structurally similar aromatic dye Congo red and that this ability is strongly correlated with virulence (15, 42). The molecular basis for this absorption is unclear. In our case no differences in the Congo red or hemin acquisition was observed by all of the strains tested regardless of the media used, indicating that this could be due to the existence of constitutive binding-molecules located at the *T. maritimum* cell surface.

This hypothesis was confirmed by using dot blot assays. It was found that whole cells of all *T. maritimum* strains tested, regardless of the culture conditions, were able to bind hemin (Fig. 3). Interestingly, when iron-limited or iron-supplemented bacterial cells were pretreated with proteinase K, their ability to bind hemin was not reduced compared to that of cells not treated with the enzyme. It indicates that, in addition to protein binding, some protease-resistant components could also bind hemin. Similar results have been reported for other gram-negative bacterial species (17, 18) in which some other components, such as capsular polysaccharides and/or lipopolysac-

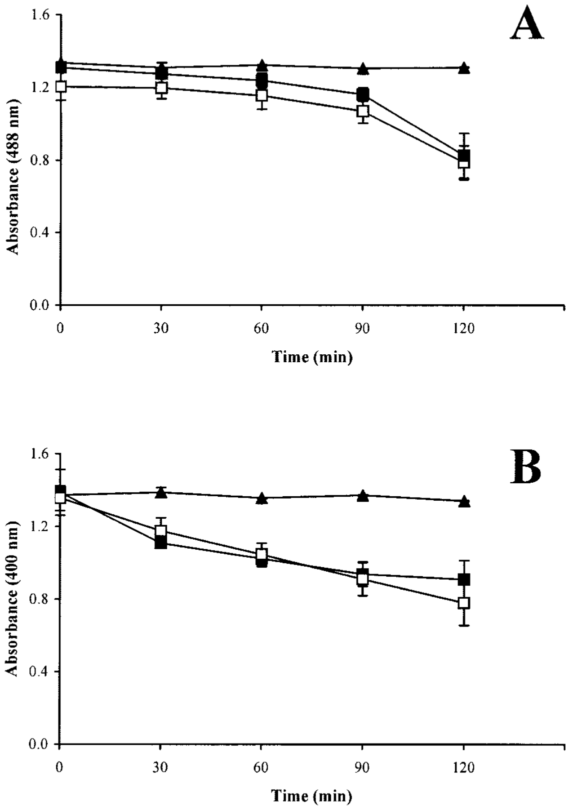

FIG. 2. Binding of Congo red (A) and hemin (B) by three *T. maritimum* isolates. Cells were cultured under either iron-restricted (■) and iron-replete (□) conditions. Negative control, Congo red, and hemin data from a cell-free flask (▲) are also shown. The uptake of Congo red and hemin are expressed here as depletion of the dye from solution as a function of time. Bars represent the standard deviation of the data from two different experiments.

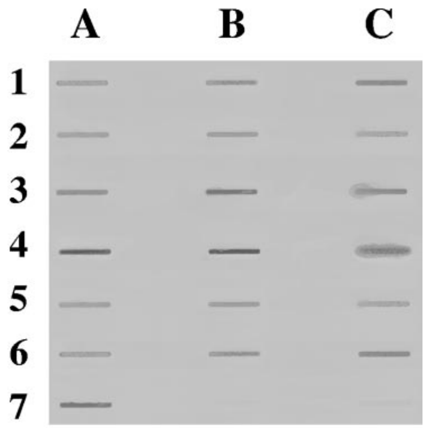

FIG. 3. Binding of hemin by *T. maritimum*. Lanes: A, strain PC503.1; B, strain PC424.1; C, strain ACC6.1. Rows: 1 and 2, whole cells cultured under iron-supplemented (FMM plus $FeCl_3$) (row 1) and iron-restricted (FMM with EDDHA) (row 2) conditions; 3 and 4, outer membrane proteins obtained under iron-supplemented (row 3) and iron-restricted (row 4) conditions; 5 and 6, effect of proteinase K on the binding activity cultured under iron-supplemented (row 5) or iron-restricted (row 6) conditions; 7, controls (positive control *V. anguillarum* 775 [lane A]; negative control *E. coli* HB101 [lane B]; proteinase K alone [lane C]).

charides, allows bacteria to bind hemin. In fact, the ability of *T. maritimum* purified lipopolysaccharides fraction to bind hemin, and thus its affinity for the compound, was clearly detected (data not shown).

Analysis of enzymatic activities. Whole cells and supernatants of the three *T. maritimum* isolates, grown in iron-rich or iron-restricted conditions, exhibited identical enzymatic profiles in API ZYM gallery, matching the typical profile described for *T. maritimum* species (positive results in the first 11 enzymatic reactions) (4, 9, 12). Moreover, none of the isolates were able to degrade starch, carboxymethyl cellulose, and esculin when the enzymatic activity of the *T. maritimum* strains were compared by using iron-rich and iron-restricted media. However, we found that the three strains failed to hydrolyze gelatin onto media supplemented with EDDHA (the incorporation of EDDHA to the medium did not affect the total growth of the *T. maritimum* strains tested), indicating that this activity is iron dependent because *T. maritimum* has been classically considered a proteolytic bacterium in normal culture conditions (4, 12, 52). This inability to produce gelatinase was due to the reduction of levels of free iron present in FMM medium, strongly suggesting that iron is required for the synthesis and/or activity of this protease. In other fish pathogens such as *A. salmonicida* it has been demonstrated the production of metalloproteases with gelatinolytic activity, which may have a broad specificity for degrading extracellular matrix component, muscle protein, and other substrates (2).

Interestingly, the pathogenicity of *T. maritimum* has been attributed to extracellular products and hemolysins (8), which could facilitate the alteration and erosion of the host tissue contributing to the colonization and invasion, as has been described in *A. salmonicida* (20), *V. anguillarum* (37), and *Yersinia ruckeri* (46). Thus, it would be reasonable to speculate that once the bacterial multiplication begins, the production of lytic enzymes, such as hemolysins, could make the heme groups or hemoglobin released from lysed erythrocytes readily available for utilization as an iron source, triggering the expression of virulence factors. However, preliminary in vitro studies about the possible regulation by iron of the hemolysins produced by *T. maritimum* showed no differences in the hemolytic activity when the cells were grown under iron-restricted and iron-replete conditions (data not shown), suggesting that hemolysins are not iron regulated in this bacterium.

Induction of outer membrane proteins. When the *T. maritimum* strains were grown under iron-restricted conditions, all of them, regardless of their serotype, showed the induction of three IROMPs with molecular masses of ca. 128, 85, and 66 kDa (Fig. 4). The induction of IROMPs has been reported in other fish pathogens such as *V. anguillarum* (13, 29, 35), *Y. ruckeri* (44), *P. damselae* subsp. *piscicida* (30), and *E. tarda* (23). At present, the function of the *T. maritimum* IROMPs is unknown, although it is tempting to speculate that some or all

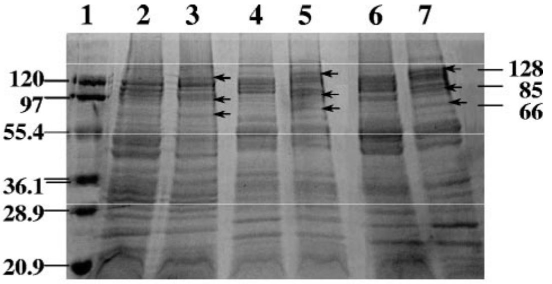

FIG. 4. SDS-PAGE analysis of outer membrane proteins of *T. maritimum* strains. Lanes: 1, molecular size markers; 2 and 3, strain PC503.1; 4 and 5, strain PC424.1; 6 and 7, strain ACC6.1. Lanes 2, 4, and 6 contained outer membrane proteins from cells cultured on FMM medium containing 20 μM $FeCl_3$. Lanes 3, 5, and 7 contained outer membrane proteins from cells grown on FMM medium containing 80 μM EDDHA. Arrows indicate the IROMPs. Numbers on the left indicate the positions of molecular size markers, and numbers on the right show the molecular masses of the IROMPs (in kilodaltons).

of these proteins may serve as receptor(s) for siderophore-iron complexes or heme groups or in some way interact directly with host iron-carrying compounds. Further experiments, including an analysis of *T. maritimum* mutants lacking IROMPs, are necessary to confirm the role of these proteins.

On the other hand, using the dot blot assay, it was found that both total and outer membrane proteins from iron-supplemented or from iron-restricted *T. maritimum* cells were able to bind hemin. The affinity for this compound was decreased by pretreatment with proteinase K. The residual binding observed was likely due to lipopolysaccharide that could be stuck to protein components during protein extraction. With the same amount of protein, stronger binding to outer membrane proteins than to total proteins was detected, indicating that a higher density of hemin-binding components is present in the outer membrane fraction (Fig. 3).

Concluding remarks. Our data clearly show for the first time that *T. maritimum* possesses at least two different systems of iron acquisition: one involving the synthesis of siderophores and another that allows the utilization of heme groups as an iron source by direct binding. Further studies will be developed in order to establish a relationship between iron uptake ability and virulence in *T. maritimum* by in vivo assays.

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VII.B. LÁMINAS COMPLEMENTARIAS A LAS PUBLICACIONES

Lámina 1. Detección de sideróforos en *Tenacibaculum maritimum* mediante el ensayo químico de Schwyn y Neilands.

Lámina 2. Cepa de *Tenacibaculum maritimum* PC503.1 crecida en condiciones restrictivas de hierro (160 μM EDDHA) y con la adición de discos impregnados con diferentes concentraciones de hemoglobina como fuente de hierro. Control corresponde al disco sin fuente de hierro.

Lámina 3. Resultados de los ensayos de inducción de la degradación de la gelatina con los productos extracelulares (ECP) y células completas (CC) de las cepas de *Tenacibaculum maritimum* PC503.1 (1-2), PC424.1 (3-4) y ACC6.1 (5-6), las cuales fueron crecida en condiciones restrictivas (80 μM EDDHA) (A) y con la adición de 20 μM $FeCl_3$ como fuente de hierro (B).

Lámina 4. Perfil electroforético de las proteínas de membrana externas de las cepas de *Tenacibaculum maritimum*. Líneas 1, Marcador de masa molecular; 2 y 3, cepa PC503.1; 4 y 5; cepa PC424.1; 6 y 7, cepa ACC6.1. Líneas 2, 4 y 6 contienen proteínas de membrana externas de células cultivadas en FMM suplementado con 20 μM $FeCl_3$. Líneas 3, 5 y 7 contienen proteínas de membrana externas de células crecidas en FMM con adición de 80 μM EDDHA. Las flechas indican IROMPs. Los números a la izquierda indican la masa molecular de los marcadores y los números a la derecha muestran las masas moleculares de las IROMPs (en kDa).

Lámina 1.

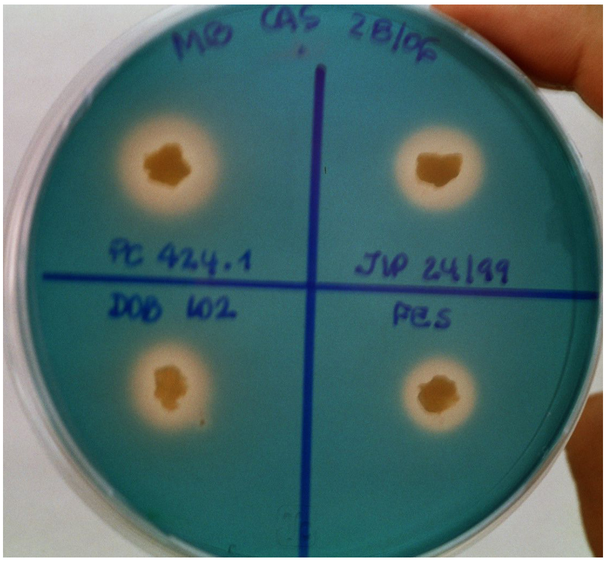


Lámina 2.

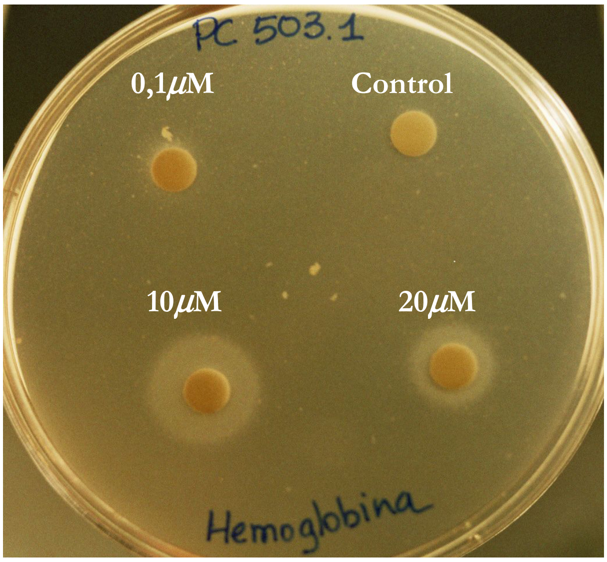


Lámina 3.

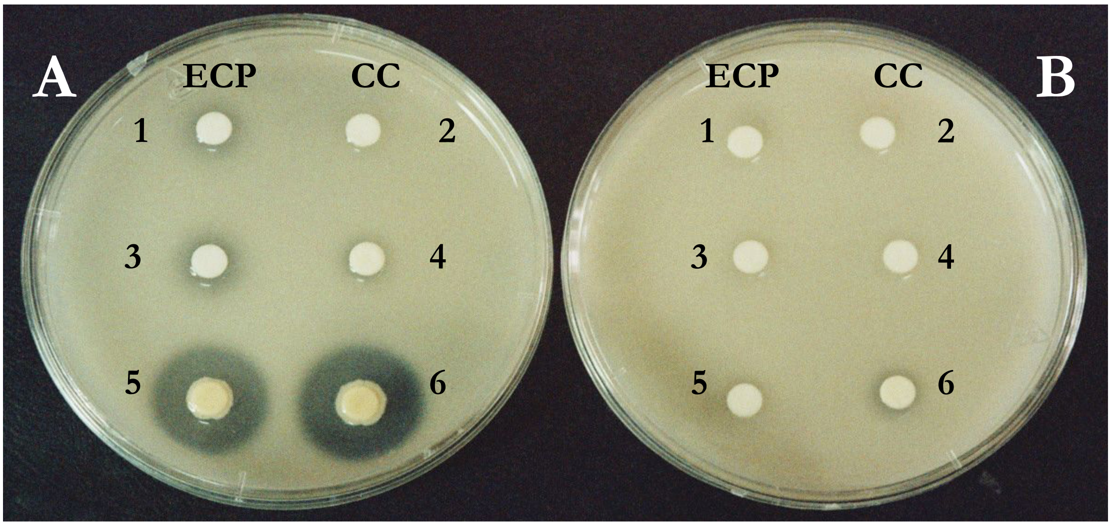
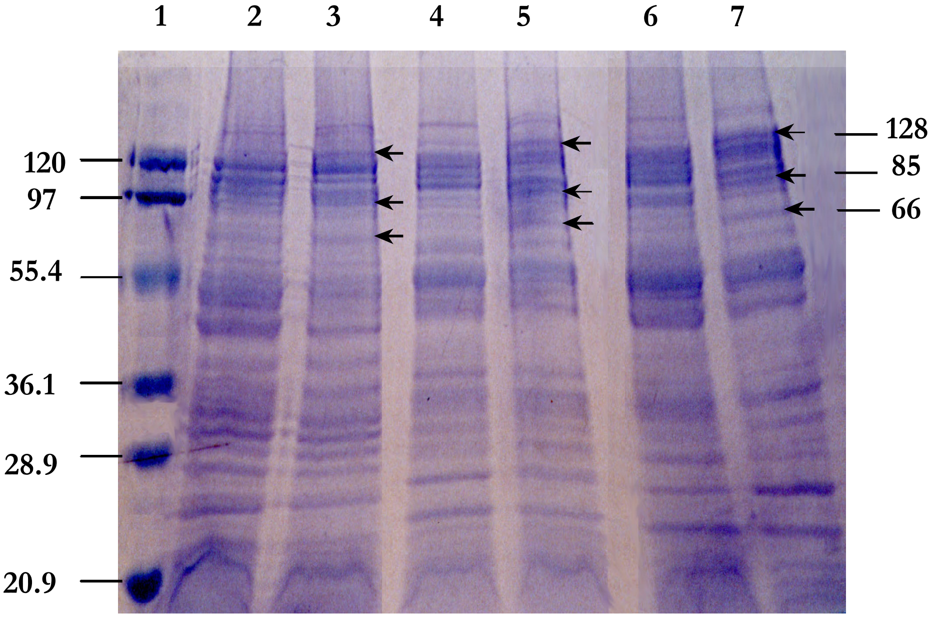


Lámina 4.



VII.C. ESTUDIOS COMPLEMENTARIOS

7.1. Determinación de la virulencia de los aislados de *Tenacibaculum maritimum*: Modelo de infección.

Hasta ahora, los estudios de patogenicidad realizados con T. *maritimum* han mostrado resultados muy diversos, dependiendo principalmente de la vía de inoculación de la bacteria y la virulencia de la misma (ver apartado 1.2.2). De esta forma, la ruta de infección de T. *maritimum* es todavía desconocida. Wakabayashi y col. (1984) han señalado que cuando los peces se exponen a una aplicación tópica de la bacteria o a una combinación de aplicación tópica e inmersión en cultivos bacterianos (Baxa y col. 1987a) se producen mortalidades sensiblemente superiores a las observadas cuando las infecciones experimentales se realizan mediante inyección intraperitoneal (Alsina y Blanch 1993). Recientemente, Powell y col. (2004; 2005) utilizando éste mecanismo de inoculación del patógeno en las branquias a concentraciones mayores a 10^{11} células por ml^{-1}, observaron mortalidades muy variables y que no pueden ser asociadas directamente a la bacteria, sino a la abrasión previa de los arcos branquiales, causando hiperplasia de los filamentos branquiales y reducción de los parámetros respiratorios (tensión arterial de oxígeno y contenido de células transportadoras de oxígeno).

En el presente trabajo, proponemos un modelo experimental de infección que permite reproducir de una manera efectiva la tenacibaculosis en rodaballo y demostramos que las cepas de T. *maritimum* pertenecientes a los diferentes serotipos podrían no tener especificidad de huésped. Además, mediante ensayos "*in vivo*" hemos puesto en evidencia que la administración a los peces de compuestos tipo hemo, junto a la inoculación de las cepas, produce un aumento significativo del grado de virulencia de los aislados de T. *maritimum*.

MATERIALES Y MÉTODOS

A. Cepas bacterianas.

El grado de virulencia para los peces se determinó utilizando las cepas de *T. maritimum* PC503.1, PC424.1 y ACC6.1, las cuales son representantes de los tres grupos serológicos mayoritarios descritos dentro de esta especie (Avendaño-Herrera y col 2004: artículo 1 y 2) (Tabla 7.1.1.). Las cepas bacterianas cultivadas en agar FMM se recogieron y resuspendieron en solución salina estéril a una concentración de 5 x 10^9 células ml^{-1} (recuento con tinción simple). El número real de bacterias viables presentes en el inóculo se determinó por siembra directa en placas de FMM de 0,1 ml de diluciones decimales de la suspensión inicial.

B. Método de infección

Las inoculaciones experimentales se llevaron a cabo en alevines de rodaballo (4 – 5 g), mantenidos en tanques (4 litros) de agua de mar y con aireación. La infección de los peces se realizó mediante inyección intraperitoneal y baño, empleando lotes de 12 peces por dosis como se describe a continuación:

Vía intraperitoneal: Se administraron 0,1 ml de las diferentes diluciones de las suspensiones bacterianas iniciales de cada aislado, de tal forma que los peces recibieron concentraciones finales comprendidas entre 5 x 10^3 y 5 x 10^7 células pez^{-1}. Como control se utilizaron peces inyectados con el mismo volumen de solución salina estéril.

Baño: Los peces fueron expuestos durante 18 – 20 h a la inoculación directa de las diferentes diluciones de cada cepa de *T. maritimum* en el agua de cultivo, de modo que las concentraciones finales en los tanques estuvieran comprendidas entre 5 x 10^3 y 5 x 10^7 células ml^{-1}. Transcurrido ese tiempo se procedió a cambiar el agua de cultivo y los peces se mantuvieron en los mismos tanques a una temperatura de 18 – 20ºC, un rango de pH entre

7,8 y 8,2; 35‰ de salinidad y aireación constante. En todos los ensayos se incluyeron como control un número igual de peces a los cuales se le adicionó en el agua de cultivo solución salina estéril y que se manipularon de la misma forma que los peces infectados.

Las mortalidades ocurridas durante el período de experimentación (21 días), se consideraron causadas por las cepas inoculadas, sólo si éstas fueron recuperadas en placas de FMM a partir del riñón de los peces moribundos y/o a partir de peces con síntomas externos característicos de la tenacibaculosis (lesiones en la boca, mandíbula o aletas). En todos los experimentos se realizó la detección de *T. maritimum* usando la técnica de PCR-anidada, tanto a partir del agua de cultivo como de los peces supervivientes (ver Capítulo V: artículos nº4 y 5).

El grado de virulencia expresado como Dosis Letal 50 (LD_{50}, número de bacterias necesarias para matar el 50% de los peces inoculados durante el período experimental) se calculó según el método de Reed y Müench (1938). Las cepas con $LD_{50}>10^7$ no fueron consideradas virulentas.

C. Efecto de un compuesto férrico en la virulencia de *Tenacibaculum maritimum*

Con el objeto de determinar el efecto del hierro en la letalidad del patógeno, un grupo de peces se inyectó intraperitonealmente con 0,1 ml de una solución de hemina a una concentración no letal de 15 μg pez^{-1}. Este grupo de rodaballos fueron infectados mediante inyección intraperitoneal y baño usando la cepa PC424.1 aislada a partir de rodaballo a concentraciones comprendidas entre 5×10^3 y 5×10^7 células pez^{-1} y células ml^{-1}, respectivamente. En todos los ensayos se incluyeron como control un número igual de peces inoculados con hemina. Las mortalidades se controlaron diariamente y se consideraron causadas por la cepa inoculada de acuerdo al mismo criterio señalado anteriormente.

RESULTADOS Y DISCUSIÓN

Los ensayos de patogenicidad en rodaballo demostraron que, independientemente de las características serológicas de las cepas empleadas y la administración de hemina, cuando en los rodaballos se utilizó el método de inoculación intraperitoneal no se pudo reproducir la enfermedad. Nuestros resultados concuerdan con los descritos por Alsina y Blanch (1993) cuando trataron de inducir también experimentalmente la infección en rodaballos, usando la propia cepa aislada a partir de una severa epizootia ocurrida en alevines de esta especie. La ineficacia de la inyección intraperitoneal como método de infección se podría explicar en base a que hemos puesto de manifiesto mediante la técnica de PCR-anidada que la mayoría de la bacteria inoculada en los peces es liberada al agua de cultivo, siendo *T. maritimum* detectado en los tejidos de los peces sólo en las primeras 18 h post-inyección.

Por el contrario, el baño prolongado de los peces con *T. maritimum* constituye un método eficaz para reproducir la enfermedad, pues dos de las cepas probadas (PC424.1 y ACC6.1) fueron virulentas para rodaballo y reprodujeron los síntomas externos característicos de la enfermedad, particularmente hemorragias en la mandíbula y boca (Fig. 7.1), desencadenando a corto plazo la muerte de los peces (entre 24 h y 10 días dependiendo de la cepa y dosis empleada). En base a los resultados obtenidos podemos concluir que la tenacibaculosis en peces se puede reproducir sin necesidad de causar la erosión o algún tipo de escarificación en la superficie externa de los peces como había sido descrito previamente para lubina (Bernardet y col. 1994).

Además, el grado de virulencia para alevines de rodaballo mediante inoculación por baño indica que no todas las cepas probadas son virulentas, siendo la cepa ACC6.1 la que presenta un valor menor de LD_{50} ($<5 \times 10^3$ células ml^{-1}) (Tabla 7.1.1.). Estudios de patogenicidad en salmón del Atlántico, trucha arcoiris y "greenback flounder" han demostrado que la exposición de los peces a dosis de *T. maritimum* entre 10^5 a 10^7 UFC ml^{-1} durante 60 a 90 minutos son suficientes para provocar la aparición de los síntomas clínicos de la tenacibaculosis, aunque con mortalidades variables (entre 7,2 a 97,5%). También, los resultados indican que no existe una relación entre el grado de patogenicidad y las características serológicas de los aislados, al mismo tiempo que no permiten suponer que dentro de las cepas de *T. maritimum* estudiadas existe especificidad de huésped, pues la cepa ACC6.1 fue virulenta para rodaballo, siendo una bacteria originalmente aislada a partir de una epizootia ocurrida en lenguado. Similares resultados han sido obtenidos por Handlinger y col. (1997), quienes infectaron truchas arcoiris usando una cepa aislada a partir de salmón del Atlántico. Sin embargo, queda por establecer si la cepa PC503.1 tiene algún grado de virulencia, y de tenerla, si se encuentra asociada al origen de aislamiento y grupo serológico, ya que en rodaballo no causó mortalidad, pero si en estudios previos cuando se utilizaron como especie de estudio alevines de lenguados (Romalde y col. 2005). Es interesante señalar que en nuestro experimento, no pudimos detectar la presencia de *T. maritimum* en los rodaballos inoculados con la cepa PC503.1, ni por cultivo tradicional ni usando la técnica de PCR-anidada.

En cuanto la administración de hierro a los peces, observamos que la hemina produjo un incremento de la virulencia del aislado de *T. maritimum* PC424.1. Esto se tradujo en la disminución de 1 unidad logarítmica de la LD_{50} y una notable reducción en el tiempo que tarda la bacteria en provocar la muerte total de los peces (72 h). Además, en todos los casos se reaisló la cepa de *T. maritimum* inoculada. Estos resultados concuerdan con lo descrito para otros patógenos de peces como *L. anguillarum* (Nakai y col. 1987), *E. tarda* (Iida y Wakabayashi 1990) y *Ph. damselae* subsp. *piscicida* (Magariños y col. 1994) donde se sugiere que el hierro es el factor responsable del aumento del grado de patogenicidad de *T. maritimum*. De hecho, es bien conocido que la incorporación de compuestos férricos antes de un ensayo de infección incrementa la letalidad de la bacteria y la susceptibilidad de los peces, por lo que podemos concluir que al menos algún mecanismo de captación de hierro participa de forma efectiva en el desarrollo de la infección.

Tabla 7.1.1. Virulencia de las cepas de *T. maritimum* para alevines de rodaballo (4 – 5 g).

Cepas	**Origen**	**Serotipo**	**Patogenicidad (LD_{50})**	
			Intraperitoneal	**Baño**[a]
PC503.1	Lenguado	O1	–	– (>5 x 10^7)
PC424.1	Rodaballo	O2	–	+ (5 x 10^4)
ACC6.1	Lenguado	O3	–	+ (<5 x 10^3)

[a] inóculo expresado en células ml^{-1}

Figura 7.2.1. Alevín de rodaballo muerto con síntomas de tenacibaculosis (boca y mandíbula hemorrágica) después de 24 h de inoculación por baño con la ACC6.1. La flecha muestra la zona afectada.

7.2. Caracterización de aislados de *Tenacibaculum maritimum*: envoltura capsular y contenido plasmídico.

Las características morfológicas y bioquímicas útiles para la identificación de *T. maritimum* han sido descritas por diversos investigadores (Tabla 2). Sin embargo, hasta ahora en *T. maritimum* no se han realizado prácticamente estudios encaminados a determinar algunas características morfológicas y moleculares como la presencia de envoltura capsular y contenido plasmídico. Ambas estructuras no sólo están implicadas en los mecanismos de virulencia de numerosos patógenos de peces, sino que también constituyen en algunos casos un rasgo descriptivo entre aislados de una misma especie. En el presente trabajo, demostramos la existencia de material capsular y la ausencia de plásmidos en los diferentes aislados de *T. maritimum* estudiados, dicha información podría ayudar a la caracterización de este patógeno, así como su posible contribución en futuros estudios de patogenicidad.

MATERIALES Y MÉTODOS

A. Cepas bacterianas.

Las veinte cepas de *T. maritimum* utilizadas en este trabajo han sido aisladas a partir de epizootias ocurridas en los cultivos de dorada, lenguado y rodaballo en piscifactorías españolas durante el período 1995 – 2004. Además, se han incluido las tres cepas de referencia procedentes de la Colección Nacional de Bacterias Industriales y Marinas (NCIMB, Aberdeen, Escocia). Todas las cepas fueron confirmadas como *T. maritimum* mediante la técnica de la PCR, utilizando los cebadores y programa descritos por Toyama y col. (1996). La identificación y caracterización a nivel morfológico, fisiológico y bioquímico de los aislados han sido descritas pormenorizadamente por Avendaño-Herrera y col. (2004: artículo nº 1). Las cepas de *T. maritimum* se cultivaron para su uso rutinario en caldo y agar *Flexibacter maritimus* (FMM) medio (Pazos y col., 1996). La incubación se realizó a 20ºC durante 72 h. La conservación de los aislados se llevó a cabo empleando viales comerciales Cryo-billes (AES Laboratory, Francia), los cuales se someten a una congelación lenta mediante pases sucesivos a 4ºC, –30ºC y –70ºC.

B. Detección del material capsular.

La presencia de envueltas capsulares, en condiciones "*in vitro*", de aislados de *T. maritimum* se determinó usando microscopía óptica y microscopía de transmisión. Además, se incluyeron como control negativo la cepa no patógena de *Photobacterium damselae* subsp. *piscicida* (EPOY-8803-II) carente de material capsular y, como control positivo, una cepa de *Lactococcus garvieae* (Y-T3) que posee cápsula constitutiva (Ravelo 1994).

Para la observación del material capsular mediante microscopía óptica se siguió la metodología descrita por Zorrilla (2000). Para ello, las cepas de *T. maritimum* se recogieron en tampón fosfato salino (PBS), se lavaron 2 veces por centrifugación (12.000 x*g* por 3 min) y se resuspendieron en el mismo tampón a una concentración de 10^6 células ml^{-1}. Posteriormente, la suspensión bacteriana se mezcló con el mismo volumen (proporción 1:1) de una solución fijadora (glutaraldehido 2,5% y lisina 100 mM en PBS) durante 20 minutos a temperatura ambiente. Posteriormente, se depositaron 30 µl en un portaobjeto y se dejó secar. Para teñir las bacterias se cubrió la preparación con una solución de fucsina básica durante 3 min, decolorando a continuación con agua. Una vez seca se observó en un microscopio Olympus BX50. La presencia de cápsula se verifica si alrededor de las bacterias se observa una estructura rosa clara, la cual contrasta con el rosa oscuro del interior de la célula.

El análisis de componentes capsulares por microscopía electrónica de transmisión se realizó siguiendo la metodología descrita por Decostere y col. (1999) y en colaboración con el Dr. Lamas de la Universidad de Santiago de Compostela. En este estudio se utilizaron las cepas de *T. maritimum* PC503.1 y PC424.1, las cuales son representantes de los grupos serológicos mayoritarios descritos dentro de esta especie (Avendaño-Herrera y col 2004: artículo 1). Dado que los polímeros capsulares tienden a colapsar durante el proceso de deshidratación, se realizó una estabilización del material capsular con antisuero de las cepas de *T. maritimum* PC503.1 y PC424.1. Para ello, las suspensiones bacterianas se ajustaron a una densidad óptica de 1,8 a 540 nm y, a continuación, se trataron con el antisuero respectivo durante 1 h a 4ºC. Tras este período, cada suspensión se resuspendió en el tampón cacodilato (0,1 M, pH 7,0) suplementado con glutaraldehido al 5% (v/v) y rojo rutenio al 0,15% (p/v) y se fijaron durante 2 h a 20ºC. A continuación las bacterias se inmovilizaron por la adición de agar al 3% (p/v), se lavaron 5 veces con tampón cacodilato suplementado con rojo rutenio al 0,05% (p/v) y se fijaron posteriormente con tetróxido de osmio al 1% (p/v) durante 2 h. Tras varios lavados en el mismo tampón, las muestras se deshidrataron con acetona, se lavaron dos veces con óxido de propileno y se incluyeron en resina de baja viscosidad (Spurr). Los cortes ultrafinos se tiñeron con acetato de uranilo y citrato de plomo y se examinaron con un microscopio electrónico Philips CM-12.

C. Análisis del contenido plasmídico.

El análisis del contenido plasmídico en las cepas de *T. maritimum* se realizó mediante la desnaturalización del ADN cromosómico por la acción del detergente alcalino dodecil-sulfato-sódico (SDS) a elevadas temperaturas siguiendo el procedimiento de Kado y Liu (1981). Para ello, cultivos frescos de las cepas de *T. maritimum* se resuspendieron en 1 ml de PBS y se centrifugaron a 12.000 x*g* durante 5 min. El precipitado resultante se resuspendió en 60 µl de una solución de SDS al 3% (p/v) en TRIS 50 mM (pH 12,4) y se incubó a 56ºC durante 45 min. Seguidamente se añadió un volumen igual de fenol : cloroformo (1:1 v/v), se agitó hasta formar una emulsión y cada muestra se centrífugo a 12.000 x*g* durante 10 min. La fase acuosa superior, conteniendo el DNA extra-cromosómico se transfirió a otro vial y se adicionó 10 µl de solución estabilizadora [púrpura de bromocresol al 0,25% (p/v) en Tris-acetato 50 mM, pH 7,9 y glicerol al 50% (v/v)].

Sin embargo, el método de extracción de plásmidos descritos por Kado y Liu (1981) a menudo genera un mayor número de bandas próximas del ADN cromosómico, por lo cual las cepas de *T. maritimum* también fueron examinadas usando el método descrito por Birnboim y Doly (1979) modificados por Sambrook y col. (1989). La base de esta técnica consiste en la desnaturalización del ADN cromosómico mediante lisis alcalina. En este caso, los cultivos bacterianos en caldo de FMM se transfirieron a viales Eppendorf y se centrifugaron a 10.000 x*g* durante 5 min. El precipitado resultante se resuspendió en 200 µl de solución de lisis (glucosa 5 mM, EDTA 10 mM, TRIS-HCl 25 mM; pH 8,0) con la adición de lisozima a una concentración de 5 mg ml^{-1} y se incubó a temperatura ambiente durante 5 min. Seguidamente se añadieron 200 µl de una solución de SDS al 10% (p/v) en NaOH 10 N, se mezcló por inversión y se mantuvieron en hielo durante otros 5 min añadiendo a continuación 150 µl de acetato sódico 3 M (pH 4,8). Después de 30 min en hielo, las muestras se centrifugaron a 10.000 x*g* durante 10 min, se transfirió el sobrenadante a un nuevo vial y se centrifugó de nuevo durante 2 min. El sobrenadante obtenido se transfirió a otro vial y se adicionó a ambos tubos un volumen de isopropanol equivalente a dos veces el del sobrenadante, se esperó 15 min a temperatura ambiente y se centrifugó nuevamente. El sobrenadante se desechó y el precipitado así obtenido se secó y se resuspendió en 40 µl de tampón TAE (Tris-acetato-EDTA: Tris 6,05 M, Na_2EDTA 0,005 M, NaCl 0,05 M, pH 8,0) mezclándolo con 10 µl de la solución estabilizadora [púrpura de bromocresol al 0,25% (p/v) en Tris-acetato 50 mM, pH 7,9 y glicerol al 50% (v/v)].

Las muestras obtenidas con los dos procedimientos de extracción se sometieron a electroforesis horizontal en geles de agarosa a una concentración de 0,8% (p/v), utilizándose una intensidad constante de 100 Voltios hasta que el frente se encontrara aproximadamente a 1 cm del final del gel. El revelado de los geles se realizó por tinción con bromuro de etidio a una concentración de 0,5 μg ml^{-1} y se visualizó digitalmente con un transiluminador Gel Doc 100 (Bio-Rad) con luz ultravioleta de 254 nm.

Como marcadores de peso molecular se han utilizado los plásmidos presentes en la cepa *Escherichia coli* V517 [con masas moleculares comprendidas entre 35,8 y 1,4 Megadaltons (Md)] y en la cepa de *L. anguillarum* 775 (masa molecular de 47 Md), extraídos en las mismas condiciones que los plásmidos de los aislados de *T. maritimum.*

RESULTADOS Y DISCUSIÓN

Este es el primer estudio que describe la presencia de material capsular alrededor de las células de *T. maritimum*, la cual fue evidenciada mediante los dos procedimientos empleados. Al microscópico óptico, las preparaciones de todas las cepas con cápsula mostraron una zona transparente o halo rodeando a las células bacterianas, mientras que la cepa de *Ph. damselae* subsp. *piscicida* empleada como control negativo no presentó esta zona transparente. Cabe destacar, que el tamaño del halo no fue igual en todos los aislados estudiados, coincidiendo con la diferenciación descrita por Ravelo (2004), quien empleó la misma técnica con cepas capsuladas de la bacteria Gram positiva *L. garvieae.*

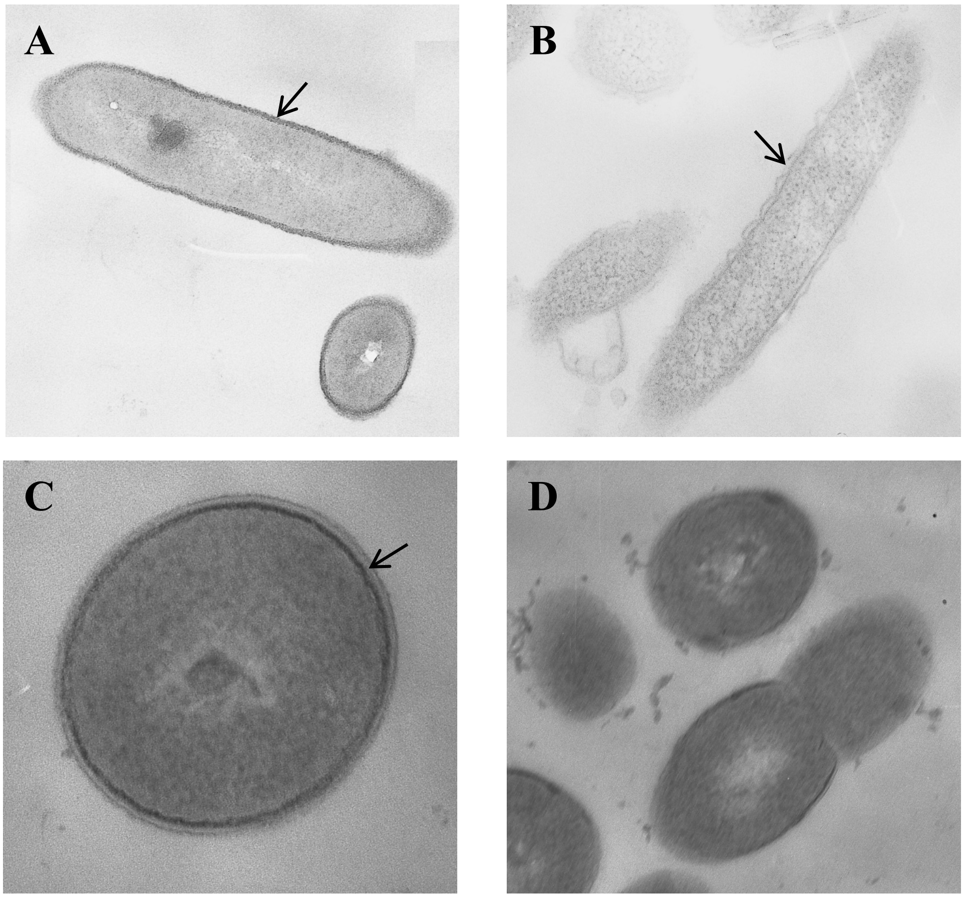
El análisis de las secciones ultrafinas mediante microscopía electrónica de transmisión de las células de *T. maritimum* estabilizadas con el antisuero de la cepa PC424.1 y PC503.1 confirmó la presencia de envoltura capsular, la cual aparece como una estructura exterior a la membrana externa de las bacterias y está fuertemente teñida con una densa capa de rojo de rutenio (Fig.7.2.1). Por el contrario, *Ph. damselae* subsp. *piscicida* no reveló la presencia de material capsular.

Resultados similares han sido señalados por Decostere y col. (1999), quienes distinguieron y asociaron el grosor de la envoltura capsular con la mayor o menor virulencia en cepas de la bacteria filamentosa patógena de peces *Flavobacterium columnare*. Estos autores también relacionaron la presencia de cápsula con la adherencia y capacidad aglutinante de la bacteria. Es interesante destacar que la capacidad de los aislados de *T. maritimum* para aglutinar eritrocitos de rodaballo y humanos ha sido demostrada previamente, así como la existencia de diferentes adhesinas responsables de las capacidades hemaglutinantes (Pazos 1997). Sin embargo, hasta ahora no existen estudios que relacionen estas características morfológicas y/o estructurales con el grado de patogenicidad de diferentes aislados de *T. maritimum* generando la necesidad de mayores estudios, para así poder establecer un claro esquema de virulencia para cepas de *T. maritimum*.

Con respecto al análisis del contenido plasmídico usando los métodos de Kado y Liu (1981) y Birnboim y Doly (1979) modificados por Sambrook y col. (1989) ninguno de los aislados de *T. maritimum* estudiados presentaron ADN extra cromosómico, lo que concuerda con lo descrito por Pazos (1997). Por otra parte, es interesante destacar que el método de Kado y Liu (1981) permite analizar un número mayor de cepas en menor tiempo. Esta misma observación ha sido descrita por Madsen y Dalsgaard (2000).

En base a los resultados obtenidos podemos concluir que la presencia de material capsular y ausencia de contenido plasmídico en los aislados de *T. maritimum* constituye un conocimiento de gran valor para estudios de caracterización morfológica y molecular de esta especie. Serán necesarios estudios mas amplios para determinar la importancia de la cápsula y su influencia en las características de la superficie celular y sobre la virulencia de este patógeno.

Figura 7.2.1. Fotografías en microscopio electrónico de secciones ultrafinas de las cepas de *Tenacibaculum maritimum* PC503.1 (A) y PC424.1 (B), así como las cepas incluidas como control negativo EPOY-8803-II (D) y control positivo Y-T3 (E). La flecha muestra una estructura fuertemente teñida con una densa capa de rojo de rutenio.



VIII. MEDIDAS DE TRATAMIENTO Y CONTROL DE LA TENACIBACULOSIS

VIII. PUBLICACIONES

Artículo nº 8: Recommendation of an appropriate medium for in vitro drug susceptibility testing of the fish pathogen *Tenacibaculum maritimum*. **Antimicrobial Agents and Chemotherapy.** 49:82-87.

Artículo nº 9: Evolution of drug resistance and minimum inhibitory concentration to enrofloxacin in *Tenacibaculum maritimum* strains isolated in fish farms. **Aquaculture.** (in press).

Artículo nº 10: Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). **Aquaculture.** (submitted).

CONTENIDOS:

- Estandarización de un medio de cultivo para antibiogramas en *Tenacibaculum maritimum*:
    - Medios de cultivos evaluados.
    - Ensayos de sensibilidad a agentes quimioterápicos.
    - Determinación de la concentración mínima inhibitoria (MIC) usando el método desarrollado para uso de clínica humana E-test

- Resistencia antimicrobiana y MIC a enrofloxacina de cepas de *Tenacibaculum maritimum*:
    - Evolución de la tenacibaculosis en piscifactorias del Norte de España y Portugal.
    - Ensayos de sensibilidad a diferentes agentes quimioterápicos.
    - Determinación de la MIC a enrofloxacina de las cepas aisladas.

- Uso de peróxido de hidrógeno como método profiláctico en brotes de tenacibaculosis:
  - Ensayos de susceptibilidad "*in vitro*" usando el desinfectante contra el patógeno en agua de mar y mucus de rodaballo.
  - Experimentos de reactivación de las células de *Tenacibaculum maritimum* post-tratamiento.
  - Determinación de los niveles de toxicidad del peróxido de hidrógeno para rodaballos y conducta de los peces.
  - Efecto del desinfectante en rodaballos infectados con el patógeno.

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Recommendation of an Appropriate Medium for In Vitro Drug Susceptibility Testing of the Fish Pathogen *Tenacibaculum maritimum*

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In the present study, Anacker and Ordal agar, marine agar (MA), and *Flexibacter maritimus* medium (FMM) were compared with the dilute versions of Mueller-Hinton agar (DMHA) medium recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for their use in disk diffusion tests with *Tenacibaculum maritimum* strains and to calculate the MICs of five drugs by the Etest method. Preliminary growth tests performed with 32 strains of this pathogen on each medium revealed that all strains failed to grow on DMHA, while the remaining media supported good growth of all isolates. In the susceptibility tests, which were carried out with the other three media, all strains were resistant to oxolinic acid and were highly susceptible to amoxicillin and trimethoprim-sulfamethoxazole, showing a good correspondence with the Etest values, which ranged from 0.064 to 0.75 and 0.006 to 1.5 μg/ml, respectively. Enrofloxacin and oxytetracycline produced significantly smaller inhibition zones and MICs on MA than on the other media assayed. However, fast, clear, and well-defined zones of inhibition were displayed for all strains at 24 h of incubation only on FMM by both the disk diffusion assay and Etest. In addition, FMM prepared with commercial sea salts instead of seawater was also suitable for bacterial isolation as well as for susceptibility testing. On the basis of these results, the use of FMM to determine the in vitro susceptibility of *T. maritimum* and its inclusion in a future revision of the NCCLS M42 report are recommended.

The rapid expansion of the aquaculture industry in the last decade has increased the losses caused by systemic bacterial infections in marine fish farming throughout the world. Although vaccination procedures are used to prevent the majority of bacterial diseases (15, 22, 27), at present a wide range of antimicrobial compounds are still essential for the control of clinical cases of infection in fisheries (29, 30, 31). Several methods of in vitro drug susceptibility testing of fish pathogens have been reported, including the disk diffusion assay, broth and agar dilution procedures (32), and most recently, the Etest method (6; M. Vilariño, J. L. Romalde, C. Ribao, A. E. Toranzo, and J. L. Barja, Abstr. XIX Congr. Nacional Microbiol., abstr. 139, 2003). Of these techniques, the agar disk diffusion method has been used since the 1960s and is the most widely used method in diagnostic laboratories because it is simple to perform, it presents a high degree of reliability in terms of standardization of the drug concentration, and a single bacterial isolate can be tested with a series of antimicrobials in one experiment (19).

The filamentous organism *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (35) is the causative agent of marine flexibacteriosis, an important disease in fish farms around the world (4, 8, 10, 11, 13, 16, 23, 28, 38). Since the first reports of flexibacteriosis (18, 20), the use of nonselective and/or low-nutrient media, such as marine agar (MA) and Anacker and Ordal agar (AOA) (3) prepared with seawater, has been advocated for the isolation of seawater-dependent, slow-growing *T. maritimum* isolates from infected fish. However, although both media support the growth of *T. maritimum* strains, another medium, named *Flexibacter maritimus* medium (FMM) (24), has been proposed to be the most appropriate for the successful isolation of this species from fish samples due to its ability to allow better growth of *T. maritimum* in comparison to the growth of heterotrophic halophilic bacteria, such as *Vibrio*, *Pseudomonas*, and *Alteromonas* species, which usually overgrow the plates. In addition, these three media have also been used for the routine drug susceptibility testing of this fastidious pathogen.

Although Alderman and Smith (1) reported a tentative set of antibiotic susceptibility test protocols for use with different bacteria pathogenic for fish, *T. maritimum* was not included in that guidance document. Recently, the National Committee for Clinical Laboratory Standards (NCCLS) (21) recommended the use of two versions of diluted Mueller-Hinton agar (DMHA), previously proposed for *Flavobacterium columnare* and *Flavobacterium psychrophilum*, as the best media for the routine susceptibility testing of *T. maritimum*. This was probably due to the inclusion of *T. maritimum* in the guidelines as a member of group III (gliding, flexing, and yellow-pigmented gram-negative bacteria), together with other phenotypically similar fish pathogens of the genera *Flavobacterium*. Unfortunately, the abilities of the strictly halophilic *T. maritimum* strains to grow on these Mueller-Hinton agar (MHA) variants have not been tested.

Therefore, the primary objective of the present study was to

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TABLE 1. *T. maritimum* strains used in this study

Bacterial isolate	Host species	Origin	Yr of isolation
LR2P	Sole (*Solea solea*)	Spain	1995
PC492.1	Sole (*Solea senegalensis*)	Spain	2001
PC503.1	Sole (*S. senegalensis*)	Spain	2001
PC504.1	Sole (*S. senegalensis*)	Spain	2001
PC528.1	Sole (*S. senegalensis*)	Spain	2002
PC529.1	Sole (*S. senegalensis*)	Spain	2002
AZ203.1	Sole (*S. senegalensis*)	Spain	2001
LgH35-O3[a]	Sole (*S. senegalensis*)	Spain	2003
LgV1-04[a]	Sole (*S. senegalensis*)	Spain	2004
ACC8.1	Sole (*S. senegalensis*)	Portugal	2003
PC424.1	Turbot (*Scophthalmus maximus*)	Spain	2000
PC460.1	Turbot (*S. maximus*)	Spain	2001
PC473.1	Turbot (*S. maximus*)	Spain	2001
LD12.1	Turbot (*S. maximus*)	Spain	2001
RM256.1	Turbot (*S. maximus*)	Spain	2002
RI93.1	Turbot (*S. maximus*)	Spain	2002
ACR104.1	Turbot (*S. maximus*)	Spain	2001
RM276.1	Turbot (*S. maximus*)	Spain	2004
JIP 24/99[b]	Turbot (*S. maximus*)	Spain	1999
JIP 46/00[b]	Turbot (*S. maximus*)	Spain	2000
ACC6.1	Turbot (*S. maximus*)	Portugal	2003
PC538.1	Gilthead sea bream (*Sparus aurata*)	Spain	2002
PC560.1	Gilthead sea bream (*S. aurata*)	Spain	2002
DOB102	Gilthead sea bream (*S. aurata*)	Spain	2002
PC868.1	Gilthead sea bream (*S. aurata*)	Spain	2003
DBA4a	*Seriola quinqueradiata*	Japan	1986
SSG33	*Salmo salar*	Spain	1993
JIP 32/99[b]	Sea bass (*Dicentrarchus labrax*)	France	1999
LVDH 1577.01[b]	Sea bass (*D. labrax*)	France	2003
NCIMB 2158	Sole (*S. solea*)	United Kingdom	1981
NCIMB 2153	Blackhead sea bream (*Acanthopagrus schlegeli*)	Japan	1976
NCIMB 2154^T	Japanese sea bream (*Pagrus major*)	Japan	1977

[a] Supplied by M. A. Moriñigo, Department of Microbiology, University of Malaga, Malaga, Spain.

[b] Supplied by J. F. Bernardet, Unité de Virologie et Inmunologie Moleculaires, Institut National de la Recherche Agronomique, Jouy-en-Josas Cedex, France.

compare AOA, MA, and FMM with the officially recommended versions of DMHA medium prepared with distilled water or seawater for disk diffusion susceptibility testing with a collection of *T. maritimum* isolates. The possible replacement of seawater by commercial sea salts was evaluated with all the media used. Finally, the MICs of different drugs in the different media were determined by the Etest method.

MATERIALS AND METHODS

Bacterial strains. A total of 32 strains of *T. maritimum* were included in this study (Table 1). This collection comprises 29 strains isolated from seven different marine fish species that belong to the different serotypes and clonal lineages described for this pathogen (4, 5) and three reference strains (NCIMB 2153, NCIMB 2154^T, and NCIMB 2158) from the National Collection of Industrial and Marine Bacteria (NCIMB; Aberdeen, United Kingdom). Most of the strains were collected from epizootic outbreaks during the last 10 years and were preserved by freezing them at −70°C in Cryo-bille tubes (AES Laboratory, Combourg, France). These strains were streaked on FMM and incubated at 24°C for 72 h. Before the assay, all bacterial strains were confirmed to be *T. maritimum* by biochemical testing, serological assays, and species-specific PCR (4, 5). As recommended in NCCLS document M42-R, reference strain *Escherichia coli* (ATCC 25922) from the American Type Culture Collection (ATCC; Manassas, Va.) was included for quality control in every test run and was grown on MHA plates (Difco Laboratories, Madrid, Spain) at 22 and 35°C for 16 to 20 h. Three *F. columnare* and two *F. psychrophilum* isolates were included as growth controls on each version of DMHA medium prepared with distilled water. These strains were routinely grown on tryptic soy agar (Difco) and modified AOA (36), respectively.

Test media. The NCCLS procedure (21) was carefully followed for the preparation of all media used in this study. Dilute 0.3% Mueller-Hinton broth (Difco) with 0.9% agar (Cultimed Panreac Química S.A., Barcelona, Spain) (DMHA) and DMHA supplemented with 5% fetal calf serum (FCS; Cultek S.L., Madrid, Spain) were prepared as described by the NCCLS (21). Due to the halophilic characteristic of this bacterium, the two variants of DMHA were also prepared with seawater. FMM (0.5% peptone [Difco], 0.05% yeast extract [Oxoid Ltd., Basingstoke, England], and 0.001% sodium acetate [Sigma Aldrich Química, S.A., Madrid, Spain] supplemented with 1.5% agar [Cultimed]) and AOA (0.5% tryptone [Becton Dickinson and Co., Le Pont de Claix, France], 0.05% yeast extract [Oxoid], 0.02% sodium acetate, hydrated [Sigma], and 0.02% beef extract [Cultimed] supplemented with 1.5% agar) were prepared with seawater as the diluent, according to the original descriptions (3, 24). MA (Pronadisa, Madrid, Spain) was prepared according to the instructions of the manufacturer. In addition, to avoid dependence on the availability of natural seawater by most laboratories, as well as to facilitate standardization of the protocols, all media (except MA) were also prepared with commercial sea salts (Oxoid) (4%; wt/vol) dissolved in distilled water. All experiments were carried out with three different batches of media.

Antimicrobial disks. For disk diffusion testing, five chemotherapeutic agents used for the treatment of bacterial diseases in fish were selected. Commercial disks (Oxoid) with oxolinic acid (OA; 2 μg), amoxicillin (AMX; 25 μg), trimethoprim-sulfamethoxazole (SXT [1.25 μg/23.75 μg]; 25 μg), enrofloxacin (ENR; 5 μg), and oxytetracycline (OTC; 30 μg) were used as described for the NCCLS procedures (21).

Preparation of inoculum. Although all *T. maritimum* isolates were routinely cultured on FMM agar, the abilities of the strains to grow on all media were also examined by inoculating each strain directly on each medium. To evaluate if the initial medium used in the disk diffusion assays had any influence, three colonies grown on plates with each medium which supported good growth of the strains were used to prepare the starting inocula of all *T. maritimum* isolates. Bacterial suspensions were prepared in sterile 0.9% saline solution, and just before experimental use the absorbance at 625 nm was measured on a spectrophotometer and was adjusted to 0.08 to 0.10, as indicated in the NCCLS M42 report. Simultaneously, 10-fold dilutions were prepared and 0.1 ml of each of the different dilutions was spread onto each medium to determine the recoverability of the strains (expressed as the number of CFU per milliliter). The plates were incubated at 24°C for 48 to 72 h.

Disk diffusion testing. The NCCLS recommendations (21) for the disk diffusion assay protocol were strictly followed in the disk diffusion testing methodology used in this study. The diameter of each zone of inhibition was determined to the nearest millimeter after 24, 48, and, if necessary, 72 h of incubation. Reference strain *E. coli* ATCC 25922 was used for quality control throughout the study, as described above. All tests were carried out in triplicate, and the mean ± standard deviation was calculated.

Etest method to determine MICs. To determine the MICs, the Etest method (AB Biodisk, Solna, Sweden) was performed according to the instructions in the manufacturer's package insert. Two strains of *T. maritimum* isolated from sole (strain PC503.1) and turbot (strain PC424.1), representing the two main serotypes described for this pathogen (4), together with all reference strains, were assayed on the same media used for the disk diffusion assay. The following drugs were tested: AMX, SXT, ENR, and tetracycline (an Etest strip of OTC was not commercially available). Antimicrobial agent concentrations ranged from 0.016 to 256 μg/ml for all agents except SXT (1/19), whose concentrations ranged from 0.002 to 32 μg/ml. Three plates per isolate were incubated for up to 48 h at 24°C and examined for the formation of an elliptical zone of growth inhibition. The value printed on the strip edge at the intersection of the growth inhibition zone was recorded as the MIC for *T. maritimum*. To check the performance of the Etests, *E. coli* ATCC 25922 was included because the MICs for this strain on MHA are known; it was incubated at 22 and 35°C for 16 to 20 h.

TABLE 2. Comparison of the growth and recoverability of *T. maritimum* reference strains and isolates on each medium used for disk diffusion assays

Medium	*T. maritimum* reference strains (n = 3)			*T. maritimum* isolates (n = 29)		
	Growth by diffusion test[a]	Recoverability of inoculum (CFU/ml)[b]	Time (h) to measurement	Growth by diffusion test	Recoverability of inoculum (CFU/ml)[b]	Time (h) to measurement
MA	++	$(9.5 \pm 4.66) \times 10^7$	24	++	$(2.43 \pm 1.02) \times 10^7$	24
FMM	+++	$(4.54 \pm 0.9) \times 10^6$	24	+++	$(3.63 \pm 3.35) \times 10^6$	24
FMMSS	+++	$(2.3 \pm 0.3) \times 10^6$	24	+++	$(3.89 \pm 0.39) \times 10^6$	24
AOA	++	$(1.25 \pm 0.35) \times 10^6$	48	+	$(1.92 \pm 0.61) \times 10^6$	48
AOASS	++	$(1.79 \pm 0.48) \times 10^6$	48	+	$(1.68 \pm 0.012) \times 10^6$	48

[a] +++, confluent and well-defined growth around the inhibition zones; ++, less well-defined growth and poorly clear inhibition zones; +, very poor growth.

[b] Data are means ± standard deviations for all replicates of each strain.

Statistical analysis. Differences between zone diameters on the different media compared were tested by applying one-way analysis of variance, with a P value of 0.05 indicating statistical significance (33).

RESULTS AND DISCUSSION

The available data on antimicrobial susceptibility testing of some species of fish bacterial pathogens showed that there is no consensus on the basal medium that should be used, giving the impression that an ideal medium for susceptibility testing of fish bacterial pathogens does not exist (12). Alderman and Smith (1) pointed out that there is a pressing need to establish the appropriate medium for some species, although the NCCLS frequently suggests the use of MHA and a modification of that medium for susceptibility testing of new species of fish pathogens; this is the situation with respect to *T. maritimum*. However, no studies have shown that halophilic bacteria like *T. maritimum* are able to grow on the proposed medium, DMHA, supplemented or not with FCS.

When preliminary growth tests with the 29 isolates and the 3 reference strains of *T. maritimum* were performed by inoculating each strain directly onto each medium tested, all *T. maritimum* strains afforded good growth on MA, AOA, and FMM, as well as the versions of AOA and FMM prepared with commercial sea salts (AOASS and FMMSS, respectively). As we expected, due to the known halophilic nature of this bacterium, DMHA, with and without FCS, prepared with distilled water did not support the growth of any of the *T. maritimum* strains tested after 7 days of incubation at 24°C. This finding suggests that the two versions of DMHA cannot be recommended for use for the routine disk diffusion susceptibility testing of *T. maritimum*. In contrast, each batch of DMHA supported the growth of all isolates of *F. columnare* and *F. psychrophilum* tested, showing that it is reliable for the in vitro susceptibility testing of both fish bacterial pathogens, as proposed by Hawke and Thune (17) and Bruun et al. (9), respectively. When each version of DMHA prepared with seawater or sea salts was tested, the *T. maritimum* strains presented no or scant and poorly defined growth.

With the knowledge that MA, AOA, and FMM, as well as the versions prepared with commercial sea salts, allowed the suitable growth of all *T. maritimum* isolates, only these media were used for comparative testing of the five antibacterial agents commonly used in aquaculture for the treatment of marine flexibacteriosis. In fact, these media have been routinely used for the isolation of *T. maritimum* strains from the external lesions and internal organs of infected fish (2, 4, 10, 11, 34). Although both variants of FMM and AOA, as well as MA, were capable of providing suitable growth conditions for susceptibility testing, in the first 24 h, 36.36% of the *T. maritimum* isolates tested on AOA and AOASS grew too poorly to permit the measurement of zone diameters, and 48 h was required before the results could be read (Table 2). These results agree with the values of recoverability of *T. maritimum* on AOA and AOASS, which were less than those achieved on the other media tested (Table 2). Although the strains grew faster on MA than on AOA or AOASS, poorly defined zones around the disk were produced, leading to inaccuracies in estimations of the inhibition zone sizes. All *T. maritimum* strains displayed fast, clear, and well-defined zones of inhibition only on FMM and FMMSS after 24 h of incubation, even though the numbers of recoverable cells in the inoculum fell below the concentrations recommended by the NCCLS due to the fastidious growth of this microorganism (Table 2). In addition, these zones of inhibition remained stable during the incubation period. These advantages are convenient for rou-

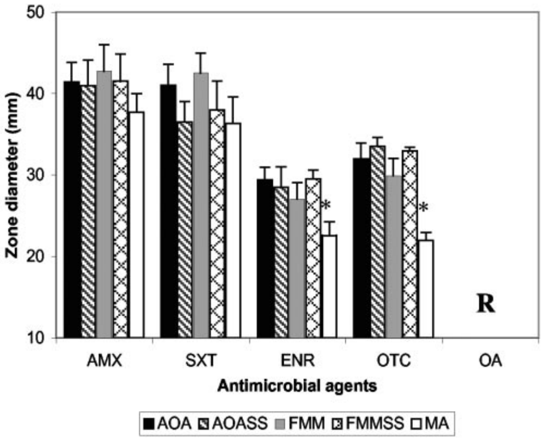


FIG. 1. Comparison of in vitro susceptibilities of 29 strains and 3 reference strains of *T. maritimum* after 48 of incubation on five difference media. All data are means ± standard deviations of three replicates in which the starting inoculum consisted of strains grown on FMM. Asterisks, significant difference ($P < 0.05$); R, resistance on all media tested.

TABLE 3. MICs of four antimicrobial agents for two *T. maritimum* isolates and reference strains determined with five different agars by Etest method

Antimicrobial agent and medium	MIC (μg/ml)				
	T. maritimum isolates ($n = 2$)	*T. maritimum* NCIMB 2154^{T}	*T. maritimum* NCIMB 2153	*T. maritimum* NCIMB 2158	*E. coli* ATCC 25922[a]
AMX					
MHA	NA[b]	NA	NA	NA	4.0–6.0
AOA	0.064–0.094	0.064–0.094	0.064	0.064	2.0–3.0
AOASS	0.094–0.125	0.064–0.094	0.125–0.19	0.094–0.19	3.0
FMM	0.094–0.75	0.125–0.19	0.064–0.094	0.125–019	1.5–3.0
FMMSS	0.064–0.125	0.064–0.094	0.125–0.19	0.094–0.125	2.0–4.0
MA	0.25	0.25–0.38	0.25	0.25	1.5–2.0
SXT[d]					
MHA	NA	NA	NA	NA	0.047–0.064
AOA	0.064–1.5	0.012–0.016	0.008–0.016	0.006	0.25–0.36
AOASS	0.023–0.032	0.016	0.016	0.008–0.016	0.094–0.38
FMM	0.094–1.0	0.012–0.016	0.016	0.008–0.016	0.125–0.25
FMMSS	0.012–0.032	0.016	0.012–0.016	0.016–0.023	1.5–3.0
MA	0.023–0.25	0.016–0.032	0.032–0.064	0.023	0.19–0.38
ENR					
MHA	NA	NA	NA	NA	0.008–0.023
AOA	1.5–3.0	0.75–1.0	2.0	1.0–1.5	3.0–6.0
AOASS	0.5–1.0	0.5–0.75	0.75–1.0	0.5–0.75	0.75–1.0
FMM	1.0–1.5	1.0–1.5	0.75–1.0	0.75–1.0	3.0
FMMSS	0.5–1.0	0.38	0.75–1.0	0.5–0.75	1.0–1.5
MA	2.0–3.0	2.0	3.0	1.5–2.0	4.0–8.0
TC[e]					
MHA	NA	NA	NA	NA	0.75–1.0
AOA	1.5	1.0	2.0–3.0	1.5–2.0	R[c]
AOASS	0.094–0.125	0.25–0.38	0.25–0.38	0.25–0.38	R
FMM	0.075–1.0	1.5	1.0–1.5	1.0	R
FMMSS	0.25–0.38	0.0094–0.125	0.25–0.38	0.25–0.38	R
MA	3.0–4.0	3.0	4.0	3.0	R

[a] Range of MICs obtained after incubation at 22 and 35°C for 16 to 20 h.

[b] NA, not applicable.

[c] R, resistant.

[d] SXT was used with trimethoprim and sulfamethoxazole at a ratio of 1/19.

[e] TC, tetracycline.

tine aquaculture operations, since a fast decision on the appropriate treatment can be established (14, 29).

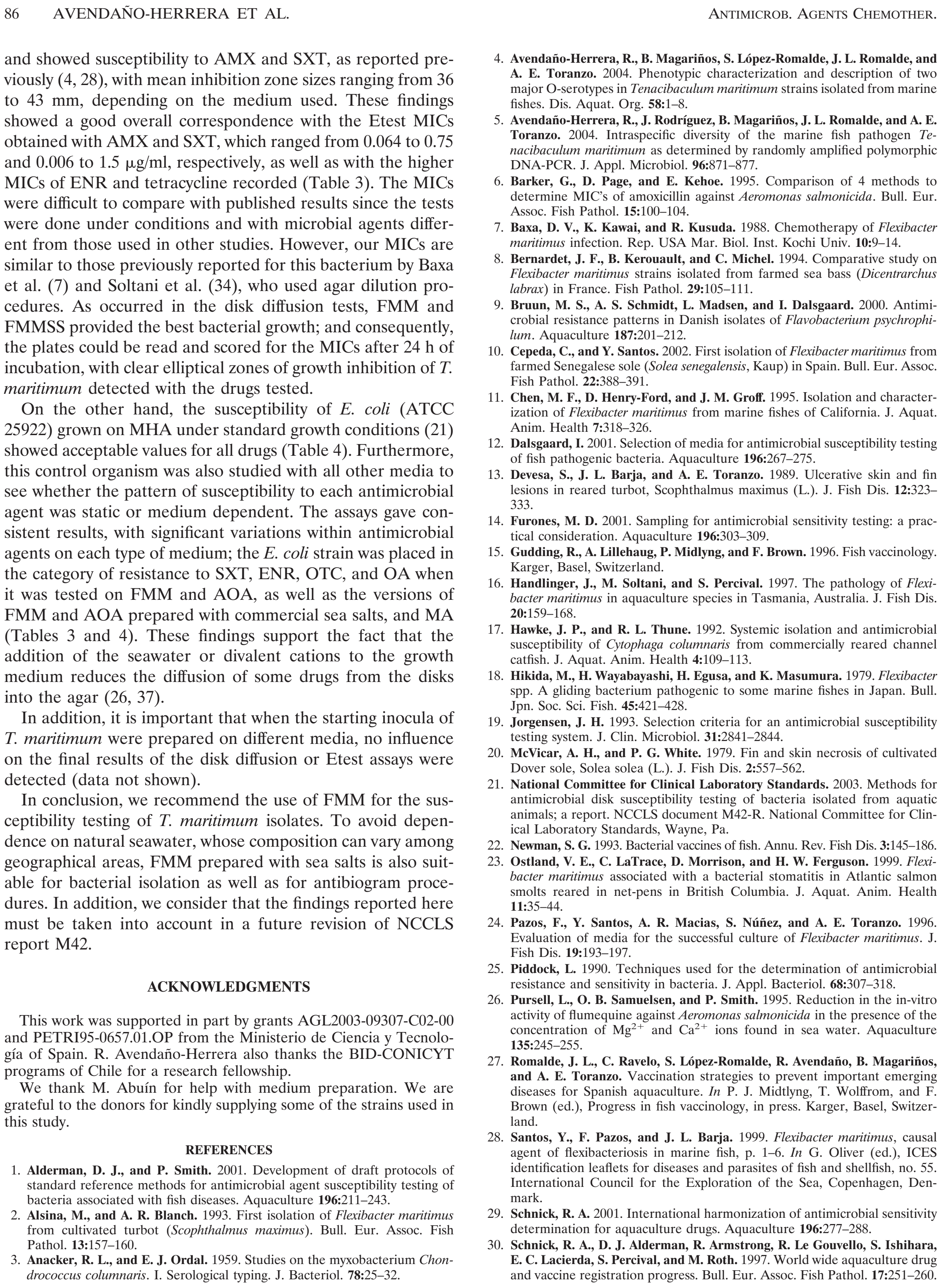
The results of the agar disk diffusion test obtained for all *T. maritimum* isolates suggested that the nature of the culture medium seems to affect the size of the inhibition zones for two of the five drugs analyzed (Fig. 1). Furthermore, the inhibition zone sizes for the 32 *T. maritimum* strains grown on MA with ENR and OTC were significantly lower ($P < 0.05$) than those on the other four media tested. This result is perhaps not surprising due to the qualitative and quantitative differences in the composition of the MA in comparison with those of the oligotrophic media, mainly in the carbon and nitrogen sources, and the presence of excessive amounts of divalent cations, which are known to affect the susceptibility testing results obtained with tetracycline in other culture media, such as tryptone yeast extract salts agar (25, 26).

Regardless of the differences between MA and the remaining media, all *T. maritimum* strains were totally resistant to OA

TABLE 4. Comparison of in vitro susceptibility testing results for quality control strain *E. coli* ATCC 25922 using MHA and five other media for each antimicrobial agent tested[a]

Antimicrobial agent	Inhibition zone diam (mm)					
	MHA	AOA	AOASS	FMM	FMMSS	MA
AMX	23 ± 1.19	24 ± 0	23.56 ± 2.73	31.33 ± 2.31	30.08 ± 2.42	27.33 ± 1.15
SXT	29.12 ± 1.55	0	0	0	0	0
ENR	37.87 ± 1.23	20 ± 0	19.06 ± 2.05	20.67 ± 1.15	19.23 ± 2.05	9.33 ± 1.15
OTC	23.75 ± 0.45	0	0	0	0	0
OA	22.67 ± 1.15	9.33 ± 1.15	9 ± 1.51	0	0	0

[a] *E. coli* ATCC 25922 was incubated at 35°C for 16 to 20 h. All data are means ± standard deviations.



and showed susceptibility to AMX and SXT, as reported previously (4, 28), with mean inhibition zone sizes ranging from 36 to 43 mm, depending on the medium used. These findings showed a good overall correspondence with the Etest MICs obtained with AMX and SXT, which ranged from 0.064 to 0.75 and 0.006 to 1.5 μg/ml, respectively, as well as with the higher MICs of ENR and tetracycline recorded (Table 3). The MICs were difficult to compare with published results since the tests were done under conditions and with microbial agents different from those used in other studies. However, our MICs are similar to those previously reported for this bacterium by Baxa et al. (7) and Soltani et al. (34), who used agar dilution procedures. As occurred in the disk diffusion tests, FMM and FMMSS provided the best bacterial growth; and consequently, the plates could be read and scored for the MICs after 24 h of incubation, with clear elliptical zones of growth inhibition of *T. maritimum* detected with the drugs tested.

On the other hand, the susceptibility of *E. coli* (ATCC 25922) grown on MHA under standard growth conditions (21) showed acceptable values for all drugs (Table 4). Furthermore, this control organism was also studied with all other media to see whether the pattern of susceptibility to each antimicrobial agent was static or medium dependent. The assays gave consistent results, with significant variations within antimicrobial agents on each type of medium; the *E. coli* strain was placed in the category of resistance to SXT, ENR, OTC, and OA when it was tested on FMM and AOA, as well as the versions of FMM and AOA prepared with commercial sea salts, and MA (Tables 3 and 4). These findings support the fact that the addition of the seawater or divalent cations to the growth medium reduces the diffusion of some drugs from the disks into the agar (26, 37).

In addition, it is important that when the starting inocula of *T. maritimum* were prepared on different media, no influence on the final results of the disk diffusion or Etest assays were detected (data not shown).

In conclusion, we recommend the use of FMM for the susceptibility testing of *T. maritimum* isolates. To avoid dependence on natural seawater, whose composition can vary among geographical areas, FMM prepared with sea salts is also suitable for bacterial isolation as well as for antibiogram procedures. In addition, we consider that the findings reported here must be taken into account in a future revision of NCCLS report M42.

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Evolution of drug resistance and minimum inhibitory concentration to enrofloxacin in *Tenacibaculum maritimum* strains isolated in fish farms

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Abstract

The *in vitro* susceptibility of 63 isolates of *Tenacibaculum maritimum* from 4 fish farms to 8 chemotherapeutic agents used for the treatment of bacterial diseases in fish were assessed. The results indicated that all strains were resistant to oxolinic acid and susceptible to amoxicillin, nitrofurantoin, florfenicol, oxytetracycline and trimethoprim-sulphamethoxazole. However, some isolates presented resistance to enrofloxacin and flumequine, ranging from 10 to 30%, and from 25 to 60%, respectively, depending on the farm sampled. These data were used in an attempt to predict whether the resistance to enrofloxacin was static or evolved during the time of sampling from 2003 to 2004. A relationship between the use of enrofloxacin and levels of resistance was detected in the studied farm, increasing significantly from no resistant isolates in 2003 to 44.8% resistant strains in 2004, the year in which this drug was commonly employed. This result was accompanied by a marked decline of about 29.2% of the inhibition zone sizes for the *T. maritimum* strains in comparison to the initial values (average 21.5 mm). Minimum inhibitory concentration (MIC) of enrofloxacin for 100 *T. maritimum* strains was determined by the microdilution method of the National Committee for Clinical Laboratory Standards (NCCLS). Twenty isolates were resistant to enrofloxacin (>256 µg ml^{-1}), while the remaining strains showed a bimodal distribution which ranged from 0.5 to 32 µg ml^{-1}. Our interpretation of the enrofloxacin MIC data suggests that the breakpoint for *T. maritimum* should be 4 µg ml^{-1}. However, similar studies in other laboratories are necessary to validate this breakpoint value.

Keywords: Tenacibaculum maritimum; Enrofloxacin; MIC; Bacterial resistance

1. Introduction

Tenacibaculosis is a serious bacterial disease affecting marine fish farming in many parts of Europe. The etiological agent of this disease is *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (Suzuki et al., 2001), a Gram negative and filamentous bacterium, which directly attacks the body surface of the fishes, causing ulcerative skin lesions, necrosis, eroded and hemorrhagic mouth, frayed fins and tail rot (McVicar and White, 1979; Baxa et al., 1986). In Spain, the first disease cases were described in turbot (*Scophthalmus maximus*), Atlantic salmon (*Salmo salar*), and lately, in sole (*Solea senegalensis* and *Solea solea*) and gilthead seabream (*Sparus aurata*) (Devesa et al., 1989; Pazos et al., 1993; Cepeda and Santos, 2002; Avendaño-Herrera et al., 2004a). Although a vaccine for turbot has been developed and tested as a preventive management tool for this disease (Toranzo et al., 2004), at present antimicrobial therapy is still necessary for the control of clinical cases on farms.

Laboratory and field trials have shown that the administration of amoxycillin and trimethoprim is an effective antimicrobial therapy against *T. maritimum*, producing adequate serum levels when given by oral or immersion procedures such as in Atlantic salmon and rainbow trout (Soltani et al., 1995). However, during the last few years, enrofloxacin (1-cyclopropyl-7-(ethyl-1-piperazinyl)-6-fluoro-1,4dihydro-4-oxo-3quinoline- carboxylic acid), a member of the 4-quinolone group with broad spectrum activity, has received growing attention for its potential in fish therapy against infections caused by bacterial pathogens such as *Aeromonas salmonicida*, *Listonella anguillarum* and *Renibacterium salmoninarum* (Dalsgaard and Bjerregaard, 1991; Stoffregen et al., 1993; Bowser et al., 1994; Hsu et al., 1994; Burka et al., 1997, Williams et al. 1997).

In the case of *T. maritimum*, data on the use of this drug is scant, even though most farmers have been administrating enrofloxacin by oral dosage incorporated in fish feed or bath as the choice for treatment of tenacibaculosis. Despite the fact that enrofloxacin is potentially active against *T. maritimum*, to our knowledge no studies to establish the minimum inhibitory concentration (MIC) values following the guidelines from the National Committee for Clinical Laboratory Standards (NCCLS) (now Clinical Laboratory Standards Institute, CLSI) have been developed and, therefore, the effect of a repetitive therapy with the same antimicrobial agent is still unknown. Considering that a selective effect of antimicrobial use on the emergence of resistant fish bacteria has been documented in several reports (Smith et al., 1994; Alderman and Hastings, 1998), a further objective was to track the possible evolution of tenacibaculosis in fish farms from the western Iberian peninsula since 2003, focusing on enrofloxacin resistance in *T. maritimum* and the characteristics of these episodes. In addition, the MIC was determined for this compound in a number of isolates of *T. maritimum* by the microdilution method.

2. Material and methods

2.1. Fish samples

Diseased turbot and sole were collected from 21 episodes which occurred in four farms located in northwest Spain and Portugal from 2003 to 2004 (Table 1). Fish with body weights ranging between 2 and 150 g were sent on ice from fish farms to the laboratory of the University of Santiago de Compostela (Spain), where they were subjected to bacteriological examination (agar cultivation and biochemical tests) and analyzed by a species specific polymerase chain reaction (PCR).

2.2. Bacteriological sampling and processing

Samples from kidney and external lesions of several fish from each outbreak were directly streaked onto plates containing marine agar (MA) (Pronadisa, Madrid, Spain) and *Flexibacter maritimus* (FMM) agar (Pazos et al., 1996). To avoid the loss of bacterial isolates, due to the slow growth characteristic of *T. maritimum*, samples were also inoculated into FMM broth. All the inoculated media were incubated at 24ºC for 2 to 5 days. Moreover, smears from skin samples and ulcers were examined using a light microscope at 400 x magnification. A representative number of the different colonial morphotypes growing on each medium were collected from plates and further purified in order to carry out identification.

2.3. Bacterial isolates

Pure cultures of the presumptive *T. maritimum* isolates, obtained by repeated plating, were subjected to identification by biochemical tests, according to the methods of Avendaño-Herrera et al., (2004a). Additionally, other non-*T. maritimum* isolates were characterized using morphological, physiological and biochemical plate and tube tests following the criteria described in the Bergey’s Manual of Systematic Bacteriology

(Holt et al., 1994) and by the API 20E systems (bioMèrieux, Madrid, Spain) (Hansen and Sorheim, 1991).

A total of 63 *T. maritimum* isolates were obtained from diseased fish from 21 outbreaks detected in the four farms. The identification of these strains was confirmed using the PCR-based analysis described by Toyama et al., (1996) and were used in the antimicrobial susceptibility testing. In addition, for the determination of enrofloxacin MIC, another 34 *T. maritimum* strains isolated from seven different marine fish species, which belonged to the different serotypes and clonal lineages described within this pathogen (Avendaño-Herrera et al., 2004 a, b), and three reference strains (NCIMB 2153, 2154^T and 2158) from the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) were also employed (Table 1). Stock cultures of all isolates were maintained frozen at –70ºC in Cryo-bille tubes (AES Laboratory, Combourg, France).

2.4. Antimicrobial susceptibility testing

Sensitivity to antimicrobial agents was evaluated by disk diffusion susceptibility testing on FMM plates, which was prepared using seawater as diluent according to the original descriptions (Pazos et al., 1996), and due to the known strict halophilic nature of this bacterium (Avendaño-Herrera et al., 2005). Eight chemotherapeutic agents used for the treatment of bacterial diseases in fish were selected. Commercial disks (Oxoid Ltd., Basingstoke, Hampshire, England) with enrofloxacin (5 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg), trimethoprim-sulphamethoxazole (1.25/23.75 µg), amoxicillin (25µg), flumequine (30 µg), florfenicol (30 µg) and oxolinic acid (2 µg) were used as described by NCCLS procedures (2003). The diameter of each zone of inhibition (point at which no growth is visible) was read twice at right angles by measuring to the nearest millimeter after incubation at 24ºC for 48 to 72 h. All tests were carried out in duplicate and the means ± standard deviation were calculated. A strain was considered as resistant to a specific drug when no growth inhibition was observed around the disk. The resistance percentage was then calculated by correlating the number of total *T. maritimum* strains isolated from each farm with the number of isolates resistant for each agent in the same farm.

Reference strain *Escherichia coli* (ATCC 25922) from the American Type Culture Collection (Rockville, MD, USA) was included for quality control throughout the study and was grown on Mueller-Hinton plates (Difco Laboratories, Madrid, Spain) at 22 and 35ºC for 16 to 20 h.

2.5. Monitoring of enrofloxacin resistance over time

Following determination of *in vitro* susceptibility testing, one farm (farm 3) with a high number of outbreaks caused by *T. maritimum*, as well as with repetitive enrofloxacin treatment just to diseased fish (30 ppm bath for 1 h during 3 days and if necessary one week) was chosen to determine whether the resistance to this agent was static or evolved during the time of sampling.

2.6. Estimation of enrofloxacin MIC

The MIC was determined using the broth microdilution testing method as described in NCCLS standard M31-A2 (2002), with some modification in the medium composition, incubation time, and temperature as required for *T. maritimum* (Avendaño-Herrera et al., 2005). One hundred strains of *T. maritimum* were evaluated in duplicate (Table 1). Broth microdilution tests were performed with 96 pre-sterilized flat-bottom microtitre plates (Nunc^{TM}, Roskilde, Denmark). Stock enrofloxacin solutions (Sigma Aldrich Química, S.A., Madrid, Spain) were prepared with solvent according to the recommendations made by NCCLS, and diluted in FMM broth. Each plate included positive controls (bacteria without enrofloxacin), negative controls (medium and sterile 0.9% saline solution), and serial two-fold dilutions of the drug. Antimicrobial containing wells included the final concentration of enrofloxacin, ranging from 0.016 to 256 μg ml^{-1}. Then, all wells except those acting as sterility controls were inoculated with 100 μl of a *T. maritimum* suspension containing approximately 5 x 10^5 CFU ml^{-1}. The final volume of each well was 200 μl. Microtitre plates were incubated at 22ºC for 24-28 and 44-48 h in stacks of no more than two panels high to ensure proper humidity and air circulation.

To check the performance of the microdilution testing, *E. coli* (ATCC 25922) was included because the MICs for this strain are known. It was incubated at both 22ºC for 24-28 h and 44-48 h, and at 35ºC for 16-20 h in cation supplemented Mueller-Hinton broth.

The MIC for each strain was determined as the last well or last dilution for which absence of growth of the microorganism was detected with the unaided eye, as well as when compared to the negative control well. In addition, the microtitre plates were read with a spectrophotometer Multiskan Plus (Version 2.01) in order to compare any possible difference in sensitivity between both reading methods.

2.7. Definition of MIC resistance and susceptibility

In vitro resistance was determined from the MIC values alone as described by Bruun et al. (2000). They state that this way of looking at resistance of *T. maritimum* isolates can be used if the MIC values fall into one or more separate groups, and then the group with the lowest MIC is defined as susceptible, and the other group as resistant assuming they have acquired some kind of a resistance determinant.

2.8. Statistical analysis

Differences between zone diameters on the different media compared were tested by applying one-way analysis of variance with a P-value of 0.05 (Sokal and Rohlf, 1980).

3. Results and discussion

Numerous studies have been reported on the incidence of pathogens affecting cultured fish species (Toranzo et al., 2005). However, until now only a few studies on the description of pathological episodes provoked by the fastidious fish pathogen *T. maritimum* have been carried out. In the present work, we have performed a characterization of the *T. maritimum* strains responsible for epizootic outbreaks in turbot and sole farms from the western Iberian peninsula since 2002, focusing on the development of antimicrobial resistance following the use of antimicrobial agents for the treatment of fish infections.

Regardless of the fish farm, the number and distribution by season of tenacibaculosis outbreaks was clearly similar in the different years. The highest number of outbreaks was detected during the spring-summer period (19), while only one outbreak was observed during autumn and winter. In fact, high water temperature has been reported as one of the crucial factors in the expression of pathogenicity by this microorganism (Handlinger et al., 1997; Santos et al., 1999). Although there were differences between the tenacibaculosis outbreaks occurring in the farms studied during the sampling period (Table 1), it is possible that the quantitative differences observed among the four fish farms could be a reflection of the different management conditions.

Besides the fact that all fish were affected, the highest occurrence and a more severe form of the disease was found in the group with body weights ranging from 2 to 80 g. This is consistent with the apparently greater susceptibility of these 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. Moreover, in the current study these open lesions were frequently surrounded by saprophytic organisms such as ciliated protozoa and bacteria, in agreement with the findings in other studies (McVicar and White, 1979; Devesa et al., 1989). Clinical observations were made on the turbot and sole specimens which showed the classical symptoms of tenacibaculosis. Microscopic examination of smears from skin lesions of these symptomatic fish revealed, in the majority of the samples the presence of abundant long, thin, rod-shaped bacteria with the ability to flex, and other motile bacteria were also seen.

Sixty three *T. maritimum* strains were recovered and characterized from turbot (17) and sole (4) outbreaks detected in the four farms. These strains were isolated from kidney (66.7%), hemorrhagic mouth (16.4%), skin lesions (14.4%) and other origins such as fins, liver, spleen and gill tissues (2.5%). In addition, the highest number of *T. maritimum* in pure culture were recovered on FMM plates from kidney samples (22 isolates), indicating that a severe septicaemia sometimes affected fish. Colonies on FMM agar exhibited the typical features for this species: flat, pale-yellow with uneven edges and strong by adherent to the medium. However, in most of the external samples (61.5%), other bacterial species were also recovered on FMM and MA plates, in agreement with the characteristic slow growth on specific synthetic media of this fastidious pathogen, that is normally overgrown by most of the species coexisting in external samples (Pazos et al., 1996; Avendaño-Herrera et al., 2004c). These isolates were identified as species of the *Vibrio* genus (84.4%), while other microorganisms belonging to *L. anguillarum, A. salmonicida* and motile *Aeromonas* species were occasionally isolated (15.6%).

Our results from the *in vitro* study on susceptibility of all *T. maritimum* isolates among the sampled farms to different antimicrobial compounds are summarized in Table 2. Regardless of the farm sampled, all *T. maritimum* strains exhibited a similar pattern, being resistant to oxolinic acid and susceptible to amoxicillin, nitrofurantoin, florfenicol, oxytetracycline and trimethoprim-sulphamethoxazole, as reported previously (Avendaño-Herrera et al. 2004a; 2005). However, some isolates presented resistance to enrofloxacin and flumequine, ranging from 10 to 30%, and from 25 to 60%, respectively, depending on the farm sampled. The rapid and combined appearance of resistance to quinolones caused by the use of these drugs in fish farms has also been described for other fish pathogens (Tsoumas et al., 1989; Toranzo et al., 1993), being mainly caused by chromosomal mutations in the gyrase genes (Baquero, 1990). Apparently, *T. maritimum* strains could be capable of acquiring resistance through this mechanism due to the fact that no plasmids or similar structures have yet been observed (unpublished data), nevertheless, further studies should be performed to investigate this phenomenon.

The results of the agar disk diffusion test obtained for all *T. maritimum* strains isolated for each farm also demonstrated that there were some significant variations in the zone sizes produced by each agent. Among the seven agents evaluated the highest coefficient of variation was detected for enrofloxacin ranging from 14.4% in farm 3 to 31.3% in farm 1 (Table 2). A comparison of the mean zone size of each drug among the four fish farms, using a single factor analysis of variance, showed significant differences in all drugs ($p<0.05$), except for oxytetracycline which displayed a mean size of 23.5 mm among the farms. When the data, calculated from the remaining drugs were compared, the largest differences were detected with amoxicillin, nitrofurantoin and trimethoprim-sulphamethoxazole and the smallest with florfenicol and quinolones (flumequine and enrofloxacin).

The susceptibility of *E. coli* (ATCC 25922) using Mueller-Hinton agar and standard growth conditions, showed acceptable values for all drugs as previously reported (Miller et al., 2003; Avendaño-Herrera et al., 2005). In the same way, MIC values obtained for this control microorganism were within the approved quality control ranges.

When the analysis of whether the resistance to enrofloxacin over time was static or evolved, differences during the time of sampling were observed in farm 3 during a 16 month period (Fig. 1). No *T. maritimum* strains resistant to enrofloxacin were detected during 2003 (year in which this drug was not used) with the average of the mean inhibition zone size being 21.5 mm. However, during 2004 a significant number (13) of the *T. maritimum* strains proved to be resistant to enrofloxacin, representing 44.8% of the total isolates during this year. In addition, the susceptible strains of *T. maritimum* recovered in 2004 showed a marked decline of about 29.2% of the inhibition zone sizes in comparison to the initial values observed in 2003. This is consistent with the relatively high amount of antibiotic therapy applied at the farm during this period, with much higher doses than those recommended for other marine fish species such as seabass (Intorre et al., 2000) and seabream (della Rocca et al., 2004). Absence of isolation of strains resistant to enrofloxacin from july to august-2004 can be explained by the temporary reduction of the use of this drug. This was done because a high resistant number of *T. maritimum* strains were recovered in the previous outbreak.

Interestingly, isolates from one outbreak often had different antibiograms, with resistant and susceptible strains being isolated from the same disease episode (Fig. 1). These findings suggest that *T. maritimum* strains with different resistance patterns could produce a succession of outbreaks over a longer period of time (Bruun et. al., 2000), since it is possible to postulate that enrofloxacin controlled the rapid proliferation of *T. maritimum* in the short term, but did not eliminate the pathogen, as has already been demonstrated in *A. salmonicida* infection (Williams et al., 1997). Therefore, selective pressure exerted by the repetitive therapy with the same antimicrobial agent could have increased the frequency of these resistant organisms within the population and further diminished the effect of chemotherapy.

The distribution of MIC values of enrofloxacin for the *T. maritimum* strains used in this study is presented in Fig. 2. In 80 strains, enrofloxacin MICs generally fell within the limits of 0.5 to 32 μg ml^{-1}, while 20 *T. maritimum* isolates were found to require higher doses to be inhibited (>256 μg ml^{-1}), so these strains were classified as resistant. This group consisted of 18 strains isolated from the 4 fish farms and 2 isolates from laboratory collection. The remaining isolates were grouped into two distinct clusters and the two MIC means were 1.1 for the susceptible (42 isolates) and 13.5 μg ml^{-1} for the resistant (38 isolates) strains, respectively. The gap between resistant and sensitive strains was significant, and no strains were classified as intermediate. On the other hand, it is important to outline that when the two incubation times were compared (24-28 and 44-48 h), minimal variability of MIC results was observed (data not shown). However, with an increase in time from 44 to 48 h incubation, there was a clear and well defined end-point. In addition, using a spectrophotometer the end-point was clear cut and sharply defined and consequently was easier to record.

On preliminary findings using the E-test method the MIC values never exceeded 1.5 μg ml^{-1} for *T. maritimum* strains (Avendaño-Herrera et al., 2005). However, the present study included more strains and the standardized methodologies outlined in the NCCLS. Our interpretation of the enrofloxacin MIC data suggests that the breakpoint for *T. maritimum* should be 4 μg ml^{-1}, which is higher than mean MIC values of enrofloxacin for the most common fish pathogens isolated from naturally infected animals that range from 0.005 μg ml^{-1} for *Y. ruckeri* to 0.032 μg ml^{-1} for *L. anguillarum* and 0.012 μg ml^{-1} for *A. salmonicida* (Dalsgaard and Bjerregaard, 1991). However, Intorre et al., (2000) noted that the majority of fish pathogens had a MIC for enrofloxacin of approximately 0.16 μg ml^{-1}. It is important to outline that all these studies were performed using Mueller-Hinton agar as the base medium. This situation differs from the current study, where FMM broth or agar prepared with seawater has been use as standard medium, which contains the divalent cations (magnesium and calcium) that are known to reduce the biological activity of quinolones (Pursell et al., 1995). Although we found an increase in MIC values for the tested *T. maritimum* strains against enrofloxacin, we consider that our finding are more similar to the field marine condition since the efficacy of a drug is related to its capacity to reach and maintain adequate concentrations at the site of infection (Intorre et al., 2000).

In summary, although in our study we complied with the NCCLS guidelines, it is still necessary that the breakpoint estimated for enrofloxacin (4 μg ml^{-1}) from MIC distribution data in *T. maritimum* should be determined in other laboratories, before being considered as a standard value for this fish pathogen. Moreover, since the efficacy of enrofloxacin against tenacibaculosis is related to its capacity to reach and maintain adequate concentrations at the site of infection, additional pharmacokinetics studies of this drug are necessary in cultured marine fish.

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Table 1. Summary of numbers and origin of the *T. maritimum* strains included in this study. NCIMB: National Collection of Marine and Industrial Bacteria (Aberdeen, UK).

Source	**Host species**	**No. of cases**	**No. isolates**	**Geographical origin**
Farm				
1	Turbot *(Scophthalmus maximus)*	4	10	Spain
2	Turbot *(Scophthalmus maximus)*	3	4	Spain
3	Turbot *(Scophthalmus maximus)*	10	39	Spain
4	Sole *(Solea senegalensis)*	4	10	Portugal
Sub-total		*21*	*63*	
Laboratory collection				
	Turbot *(Scophthalmus maximus)*		13	Spain
	Sole *(Solea senegalensis)*		10	Spain
	Sole *(Solea solea)*		1	Spain
	Gilthead seabream *(Sparus aurata)*		5	Spain
	Yellowtail *(Seriola quinqueradiata)*		1	Japan
	Atlantic salmon *(Salmo salar)*		2	Spain
	Sea bass *(Dicentrarchus labrax)*		2	France
Sub-total			*34*	
Reference strains				
NCIMB 2158	Sole *(Solea solea)*		1	United Kingdom
NCIMB 2153	Blackhead seabream *(Acanthopagrus schlegeli)*		1	Japan
NCIMB 2154^T	Japanese seabream *(Pagrus major)*		1	Japan
Total			*100*	

Table 2. Comparison of the resistance level and *in vitro* susceptibility between the sampling farms for seven antimicrobial agents tested against 63 *T. maritimum* isolates. For each agent the data generated are analyzed as a single data set. AML = amoxicillin; ENR = enrofloxacin; F300 = nitrofurantoin; FFC = florfenicol; OTC = oxytetracycline; SXT = trimethoprim-sulphamethoxazole; UB = flumequine. All strains were resistant to oxolinic acid.

		Antimicrobial agent						
Farms	Parameter	AML	ENR	F300	FCC	OTC	SXT	UB
1	Resistant (%) [a]	0	10	0	0	0	0	60
	Mean diameter (mm)	29.4	15.7	25.3	29.9	22.7	26	15.3
	Standard deviation (mm)	4	4.9	2.9	2.9	3.2	3.5	3.8
	Coefficient of variation (%)	13.7	31.3	11.5	9.8	13.9	13.3	24.8
	Maximum (mm)	34	22	30	34	28	32	19
	Minimum (mm)	25	9	20	25	19	24	10
2	Resistant (%)	0	25	0	0	0	0	25
	Mean diameter (mm)	40.3	25.7	36.3	35.5	23.8	39.3	21
	Standard deviation (mm)	8.8	4.5	6	6	5.3	8.3	4.6
	Coefficient of variation (%)	21.8	17.6	16.5	17	22.4	21.2	21.8
	Maximum (mm)	50	30	45	44	28	50	25
	Minimum (mm)	31	21	32	30	16	30	16
3	Resistant (%)	0	33.3	0	0	0	0	46
	Mean diameter (mm)	32.8	20.3	29.3	32	25.2	31.2	17.6
	Standard deviation (mm)	3.4	2.9	3.8	3.1	3.8	3.7	2.7
	Coefficient of variation (%)	10.3	14.4	12.9	9.8	14.9	11.8	15.4
	Maximum (mm)	40	27	38	38	33	39	22
	Minimum (mm)	27	16	25	25	20	24	14
4	Resistant (%)	0	30	0	0	0	0	30
	Mean diameter (mm)	30	20.3	27.3	29	22.3	30.5	17
	Standard deviation (mm)	5	3.3	4.4	5.2	3.3	5.5	2.4
	Coefficient of variation (%)	16.6	16.3	16	18	14.7	18.2	14.4
	Maximum (mm)	36	25	34	35	26	40	20
	Minimum (mm)	22	18	22	20	16	24	15

[a] The resistance percentage was calculated by correlating the number of total *T. maritimum* strains isolated from each farm with the number of isolates resistant for each agent in the same farm.

Figure Legends

Fig. 1. Evolution of the enrofloxacin resistance in *T. maritimum* strains isolated from a turbot farm during the studied period. Non-resistant (□) and resistant isolates (■). Means ± standard deviation values of the inhibition zone sizes for the resistant *T. maritimum* strains by episodes (●). Arrow indicates the beginning of the use of enrofloxacin treatment just to diseased fish (30 ppm dosage by bath for 1 h during 3 days and if necessary one week).

Fig. 2. Frequencies of MICs for *T. maritimum* isolates (*n* = 80) according to source isolation, determined by microdilution method. Arrow indicates the suggested breakpoint for enrofloxacin. The remaining *T. maritimum* isolates tested (*n* = 20) were classified as resistant (>256 µg ml^{-1}).

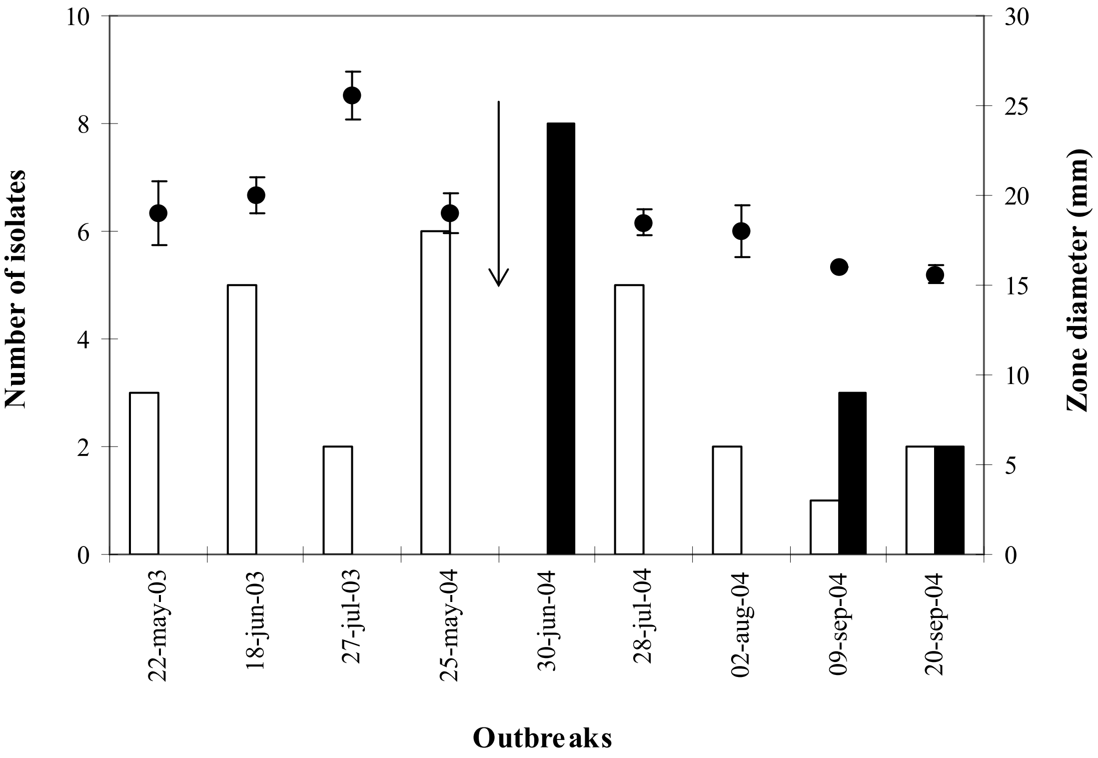

Fig. 1. Avendaño-Herrera et al.

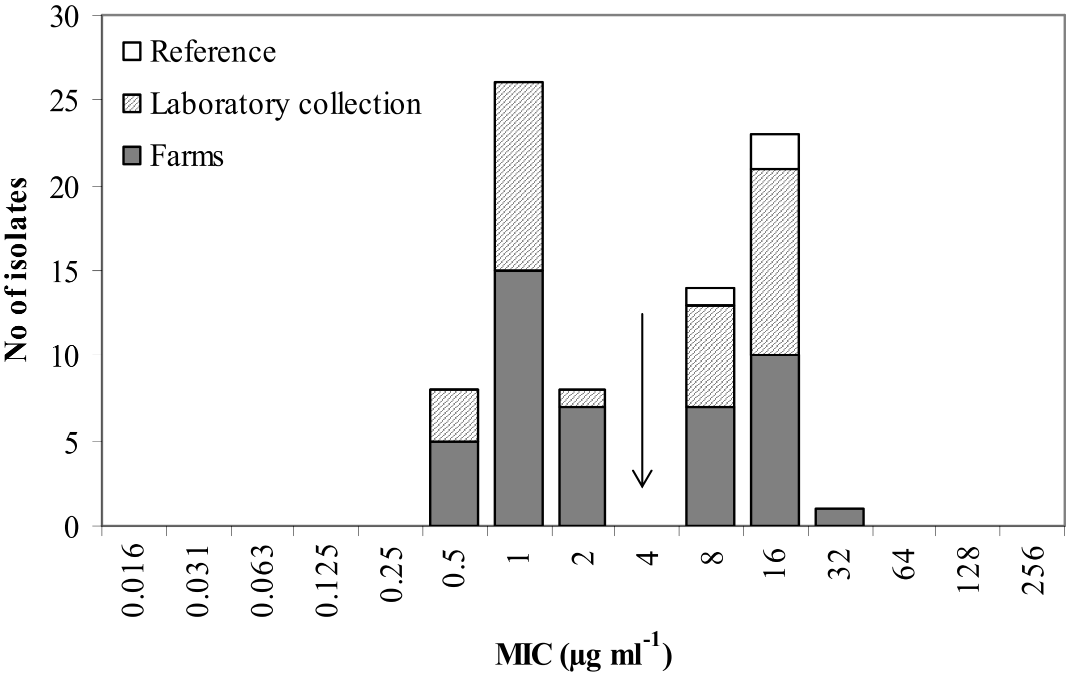

Fig. 2. Avendaño-Herrera et al.

Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*)

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Abstract

Hydrogen peroxide (H_2O_2), has recently received attention for its effective control of numerous external pathogens to fish. This study examines whether this chemical disinfectant at concentrations ranging from 30 to 240 ppm, have capacity to kill *Tenacibaculum maritimum* in *in vitro* assays. Moreover, in order to evaluate the effectiveness of H_2O_2 in infection conditions, marine tenacibaculosis was induced in turbot (8 to 10 g), *Scophthalmus maximus*, by bath exposure of 10^6 *T. maritimum* cells ml^{-1} and treated with 30 or 240 ppm H_2O_2. *In vitro* conditions, all concentrations tested were efficacious at killing a high proportion of *T. maritimum* in the seawater after 30 min of exposure. In the case of treated skin mucus, the effect of 30 ppm H_2O_2 on the bacterium was mitigated by the presence of mucus, and treatment with higher concentration of H_2O_2 (240 ppm) are needed to kill the pathogen. Besides that these concentrations were not toxic for the fish, they were not effective for the treatment of experimentally infected turbot due to the increasing levels of stress, which did not lead to death, but accelerated the tenacibaculosis outbreak. Based on our results, we recommend the use of H_2O_2 at a concentration of 240 ppm only as a general disinfection method for treating water culture and surface of tanks before the introduction of fish.

Keywords: Tenacibaculum maritimum, hydrogen peroxide, *Scophthalmus maximus*, disinfection.

1. Introduction

Marine tenacibaculosis, which is caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), is an economically important disease in a great variety of European cultured fish. This pathogen primarily attacks skin, mouth, fins and tail of fish, causing severe necrotic and ulcerative lesions on the body surface (Toranzo et al., 2005). Up to now, most treatments proposed for the tenacibaculosis outbreaks are based on the administration of drugs through feed. However, studies indicated that the effect of a repetitive therapy with the same antimicrobial agent has originated the emergence of *T. maritimum* resistant strains within the population and further diminished the effect of chemotherapy (Avendaño-Herrera et al., 2005).

An alternative therapy to the use of drugs are surface-acting disinfectants by means of bath. Formalin and iodophors are the most widely used disinfectants in European aquaculture, which have a maximum residue limit and marketing authorization in most countries (Costello et al., 2001). However, some hatchery managers have expressed concern about user sensitisation to formalin and its environment impact. In addition, this compound is expensive, difficult to use and store. On the other hand, organic iodine compounds have been recommended mainly to egg and equipment disinfection procedures (Kumagai et al., 1998; Hirazawa et al., 1999; Cipriano et al., 2001; Costello et al., 2001; Tendencia, 2001).

Another prophylactic treatment, hydrogen peroxide (H_2O_2), has recently received attention for its effective control of numerous external pathogens to fish, particularly against those of the genera *Flavobacterium* and *Cytophaga* (Lumsden et al., 1998; Derksen et al., 1999; Thomas-Jinu and Goodwin, 2004), that are phenotypically similar to *T. maritimum*. This compound, which is naturally produced during oxidative metabolism in cells, is a common bacteriostatic chemical found in most environments.

It is a potent source of reactive oxygen species (ROS) such as superoxide, perhidroxyl and hydroxyl radicals, being capable of causing lipid peroxidation, destruction of protein and DNA, and the activation and inactivation of enzymes (Gilbert et al., 1984; Afanas'ev, 1991; Vroegop, et al. 1995). Thus, bacterial pathogens must overcome the toxic effect of ROS to establish infection, protecting themselves by the production of superoxide dismutase and catalase enzymes (Díaz-Rosales et al., 2003). In addition, H_2O_2 represents a more environmental friendly alternative, because it is decomposed readily into water and gaseous oxygen (Kiemer and Black, 1997), being classified as a low regulatory compound by the Food and Drug Administration of the United States.

Until now, no therapeutic treatment based on H_2O_2 have been applied to *T. maritimum*. Therefore, the present study examines whether this chemical disinfectant have capacity to kill *T. maritimum* by *in vitro* assays. Moreover, its effectiveness for the treatment of turbot experimentally infected with this bacterium was also evaluated, in order to propose a simple, low-cost, low-technology field system to prevent and/or control tenacibaculosis outbreaks.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The *T. maritimum* strain used in this work, PC424.1, was isolated in Spain in 2000 from skin of clinically infected turbot and maintained frozen at –70ºC in Cryo-bille tubes (AES Laboratory, France). This strain belongs to serotype O2 and is included in one of the main clonal lineages (IIa) described within this pathogen (Avendaño-Herrera et al., 2004a; b). It was chosen as the representative of the *T. maritimum* species due to the fact that all the fish isolates show a high phenotypic homogeneity. For the experiment, the bacterium was routinely grown on *Flexibacter maritimus* (FMM) plates (Pazos et al., 1996) at 20ºC for 72 h.

2.2. In vitro susceptibility of T. maritimum *to* H_2O_2

To determine the effect of different H_2O_2 concentrations on *T. maritimum*, bactericidal assays were conducted with seawater taken in three different times from Ría de Muros in Galicia, NW Spain (salinity 35 ± 1‰; pH 8.0 ± 0.2). The experimental assays with each seawater sample were conducted in sterile 6-well Multidishes (Nunclon^{TM} Surface, Denmark) containing 10 ml of natural seawater. Inocula were prepared from cells scrapped off from FMM plates, washed in 0.85% sterile saline solution (SS), resuspended and diluted in SS, and then each well was seeded with 500 µl of the bacterial suspension to achieve an initial bacterial concentration of approximately 10^6 cells ml^{-1}. After an stabilization period of 30 min, susceptibility of *T. maritimum* to each H_2O_2 concentration was examined in triplicate, adding this chemical compound at final concentrations of 30, 60, 120 and 240 ppm, and then incubated at 20ºC for 24 h. Controls without the addition of the *T. maritimum* strain were run simultaneously in exactly the same conditions as described above.

To determine the evolution of culturable cells in the seawater, samples of 0.5 ml were taken aseptically, after 15 and 30 min and then 24 h post treatment. All samples were serial-diluted in SS and 0.1 ml of each dilution was plated in duplicate on Marine agar (MA) (Pronadisa, Spain) plates for the count of total heterotrophic marine bacteria. The same bacteriological analysis was carried out in duplicate on FMM agar, because it is the most effective medium for the recovery of *T. maritimum*, exhibiting the typical colonies features for this species (Pazos et al., 1996).

2.3. Resuscitation assays of the T. maritimum *cells*

When culturable cells were not detected in the experiment described above, the reactivation of *T. maritimum* to a culturable state was attempted by the addition of sterile FMM broth in a proportion 1: 1000 (v/v) to wells containing H_2O_2 at 30 and 240 ppm, as well as the control sets and incubated at 20ºC for 24 h. To confirm the absence or presence of *T. maritimum*, samples were taken at the end of incubation and were spread onto each media as described in the *in vitro* assays.

2.4. Effect of disinfectant treatments on the survival of the strain in skin mucus

Mucus was scraped off from the skin of four *T. maritimum*-free healthy turbot with a glass slide which was passed along the animal from the caudal peduncle to operculum. The assay was also conducted in sterile 6-well Multidishes containing the mucus samples diluted 1:10 (v/v) in filtered seawater, and then mixed by repeated pipetting. Each well was seeded with 500 µl of the isolate PC424.1 resuspended in SS at a final concentration of approximately 10^6 cells ml^{-1}. After an stabilization period of 30 min, H_2O_2 at 30 and 240 ppm was applied to the wells. Samples were incubated for 1 h at 20ºC and surviving bacteria were enumerated by viable counts on FMM plates. The survival of H_2O_2-treated bacteria was expressed as the percentage of colony forming unit recovered compared with untreated samples.

2.5. Toxicity of H_2O_2 to turbot

Healthy tenacibaculosis-free turbot (average weight 8 to 10 g) reared in a hatchery in the northwest of Spain were used for this experiment. Four 4-L plastic tanks, each with 10 fish, were treated for a period of 30 min with H_2O_2 at 30, 240 and 480 ppm. The remaining tank was left as untreated control. All assays were performed in duplicate. Following exposure, fish of each tank were observed to monitor fright response, respiration (rate, opercular flaring, natatorium behaviour, etc.) and latent mortality due to H_2O_2.

2.6. Effect of H_2O_2 in experimentally affected fish

Twelve tanks, each with ten healthy tenacibaculosis-free turbot (average weight 8 to 10 g) arbitrarily allocated to their experimental 4-L plastic tanks were used in this experiment. Following a 48 h acclimatization period, six tanks were challenged by direct inoculation with a *T. maritimum* suspension of 10^6 cells ml^{-1} into seawater. After a 18-h exposure, addition of H_2O_2 at a concentration of 30 or 240 ppm was performed to the tanks and incubated for 30 min. For this, two tanks were treated with 30 ppm H_2O_2 and other two with 240 ppm. Negative controls, consisted of two tanks by treatment with fish exposed to the same H_2O_2 concentrations but not inoculated with *T. maritimum.* Two groups of challenged fish but not treated with H_2O_2 were included as the positive controls. Another two tanks with fish without neither bacterial challenge nor treatment were also included. Tanks were aerated continuously, with the exception when fish were exposed to disinfectant treatment. Incubation trials were conducted in water at 18 – 20ºC with a pH ranging from 7.8 to 8.2, and the salinity 35‰ using a 16L:8D light regime.

For bacteriological analysis, water samples were taken from the 12 experimental tanks 5 min before and after fish were treated with the chemical compound. Water in each tank was changed after bacteriological sampling and then once every-other-day to remove the faecal matter.

Dead fish were removed from each tank daily and were examined to confirm if the bacterium was the cause of mortality by the following methods (Avendaño-Herrera et al. 2004c): a) the kidney and external lesions were directly streaked onto FMM plates and incubated at 20ºC for one week and b) smears from skin samples and ulcers were examined using a light microscope at 400 x magnification.

3. Results and discussion

3.1. Bactericidal effect of H_2O_2 on T. maritimum

The addition of H_2O_2 solutions to seawater, killed completely *T. maritimum* at concentrations of 120 and 240 ppm in 15 min compared to untreated seawater controls. When decreasing concentrations of H_2O_2 were used, a higher degree of resistance to the disinfectant compound was observed. However, *T. maritimum* cells were not found at 30 min for all treatments (Table 1). To clarify if this microorganism was really killed when the treatment with the disinfectant was performed, after 24 h we tried to resuscitate *T. maritimum* by the addition of fresh nutrient to each well. It is important to denote that in seawater treated with 240 ppm H_2O_2, *T. maritimum* was not recovered, indicating that the bacterium was completely killed. However, in the seawater treated with 30 ppm H_2O_2 the pathogen was recovered at values of approximately 10^3 CFU ml^{-1}, although other marine bacteria showed also a high rate of recovery (Fig.1). *T. maritimum* is a catalase positive microorganism (Suzuki et al. 2001), and thus could decompose peroxide radicals increasing survival of bacteria in presence of low concentration of peroxide as have been reported for other bacterial pathogens such as *Aeromonas salmonicida*, *Vibrio harveyi*, *Photobacterium damselae* subsp. *piscicida* (Barnes et al., 1999; Vattanaviboon and Mongkolsuk 2001; Díaz-Rosales et al., 2003). Additional studies of the role of catalase in the protection of this pathogen against peroxide should be determined. Obviously, the dose and length of exposure to H_2O_2 will determine its bactericidal or bacteriostatic effect (Hyslop et al., 1995).

3.2. Treatment of mucus with H_2O_2 after bacterial infection

The survival capacity of the *T. maritimum* strain in the mucus after the H_2O_2 treatments is shown in Fig. 2. In general, the plate count method was not capable of detecting culturable *T. maritimum* from the mucus disinfected with 240 ppm for 30 min. However, when the infected mucus was treated with 30 ppm H_2O_2, the bacterium persisted at the density of 10 CFU ml^{-1}. On the other hand, autochthonous populations of heterotrophic marine bacteria from mucus survived the exposure in all treatments, although initially they experienced a slight drop in the percentage of population of viable cells. It is well known that the primary site of infection by *T. maritimum* is the body surface, since this pathogen attaches strongly to external skin and mucus which does not contain compounds that inhibits the growth of this bacterium (Magariños et al., 1995). Therefore, *T. maritimum* can remain for a longer time in the aquatic environment utilizing fish mucus as a reservoir.

3.3. Fish sensitivity to H_2O_2 exposure

Regarding to the direct toxicity of H_2O_2 to fish, during the first minutes following the addition of 30 and 240 ppm H_2O_2, a dramatic aversion or signs of respiratory distress to treatment was demonstrated by fish. These signs included attempting to jump out of the tanks, subdued fright response, increasing ventilation rates with more rapid and marked opercula movement, and fish formed groups near the water surface. However, all fish regained normal respiratory movement and behaviour shortly after exposure to H_2O_2, therefore no mortalities occurred. By contrast, fish exposed to the greatest disinfectant concentration (480 ppm) besides displaying similar signs, inability to control position in the water and resting on the tank bottom were observed and fish mortality occurred within the first 20 h after the exposure to disinfectant.

In general, most of the H_2O_2 toxicity studies have been reported to different salmon and freshwater species, in which exposures between 50 and 500 ppm during 30 to 60 min are toxic for young fish (Rach et al., 1997, Lumsden et al., 1998, Gaikowski et al., 1999, Derksen et al., 1999, Thomas-Jinu and Goodwin 2004). These authors denote that H_2O_2 toxicity depends on the water temperature, the number and length of treatments possible, the age or life stage of the fish to be treated and water conditions as organic material and mud.

3.4. Effect of disinfectant treatment in tanks containing affected fish

With the aim to asses if H_2O_2 can be a suitable candidate for the treatment of tenacibaculosis, assays were performed in experimentally infected fish. The survival of *T. maritimum* in the seawater of the tanks exposed to 30 and 240 ppm H_2O_2 for 30 min are given in Fig. 3. With the highest concentration, there was a significant reduction of approximately 99% in the culturability of *T. maritimum* compared to untreated control. Then, standard plate counts showed that the number of culturable *T. maritimum* declined 83.9% in the tanks treated with 30 ppm. Therefore, the re-infection must be taken into account, because relatively low *T. maritimum* concentration may be quite effective to progress the disease for any length of time. In fact, the microbiological analysis on FMM plates of external skin showed recovery of this bacterium in the majority of the fish sampled. On the other hand, the autochthonous populations of marine bacteria from seawater showed a similar culturability pattern to *T. maritimum* cells but the level of survival was higher up to 30 min post-treatment.

Besides that all fish were affected by the experimentally induced *T. maritimum* infection, differences were observed depending if they were treated or untreated with H_2O_2. Both groups of treated fish (30 and 240 ppm) had a cumulative mortality of 100% within the first week post-challenged. In general, the highest occurrence and a more severe form of the disease was found in the group treated with 240 ppm H_2O_2 at the third day after the exposure. In fish that had been only inoculated with *T. maritimum*, mortality began seven days post-challenge and was 66.6 % at the end of the experiment (day 10). A similar mortality behaviour has been reported for channel catfish experimentally infected with *Flavobacterium columnare*, which infected fish treated with this compound died earlier than the infected but untreated fish (Thomas-Jinu and Goodwin 2004). As expected, no mortality was observed in the negative control groups.

These results indicate a clear association between levels of H_2O_2 used and the severity of the tenacibaculosis infection, more likely due to the increase of susceptibility of the fish favouring the entrance and spread of *T. maritimum*. It is possible that H_2O_2 treatments may result in increased levels of stress to healthy and infected fish, without causing consequent death, but also accelerate the tenacibaculosis outbreak. Probably, direct sub-acute dosing of H_2O_2 may cause destruction and necrosis of the gill epithelium as been described in other fish species (Thomassen 1993, Derksen et al., 1999). In fact, microscopic examination of smears from branchial tissues of moribund fish treated with H_2O_2 showed considerable damage and changes, trapping long, thin, rod-shaped bacteria with the ability to flex, that can then quickly colonize gills surface. In addition, observation of smears from skin lesions of all moribund fish also showed abundant presence of filamentous bacteria. On the other hand, a high number of *T. maritimum* were recovered in pure cultures on FMM plates from kidney and external mouth lesions, indicating that a severe septicaemia occurred in the affected fish.

4. Conclusion

Our results demonstrated that a 30 min exposure to H_2O_2 at a concentration of 240 ppm effectively removes a high proportion of *T. maritimum* in the seawater. However, the effect of H_2O_2 on turbot is stressful and accelerates mortality of infected fish. Therefore, we recommend the use of H_2O_2 only as a general disinfection method for treating water culture and surface of tanks before the introduction of fish.

Acknowledgements

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Table 1. *In vitro* bactericidal effect of hydrogen peroxide on *T. maritimum* in seawater.

Hydrogen peroxide concentration (ppm)	Exposure time		
	15 min	30 min	24 h
Untreated-control	–	–	–
30	±	+	+
60	±	+	+
120	+	+	+
240	+	+	+

\+ Effective (no CFU after exposure)

± Incompletely effective (Reduction of CFU after exposure)

– Sample positive for *T. maritimum* growth

Figure Legends

Fig. 1. Number of culturable *T. maritimum* strain PC424.1 (■) and other bacteria (□) after 24 h of the addition of fresh media in each well containing natural seawater and disinfected with hydrogen peroxide (30 and 240 ppm) for 30 min. Vertical bars represent standard deviations of the triplicates for each procedure.

Fig. 2. Percentage of survival of *T. maritimum* cells onto FMM plates (■) and other bacteria (□) in mucus samples after treated at different concentrations of hydrogen peroxide.

Fig. 3. Effect of hydrogen peroxide on the survival of *T. maritimum* in tanks with experimentally infected fish. Vertical bars represent standard deviations.

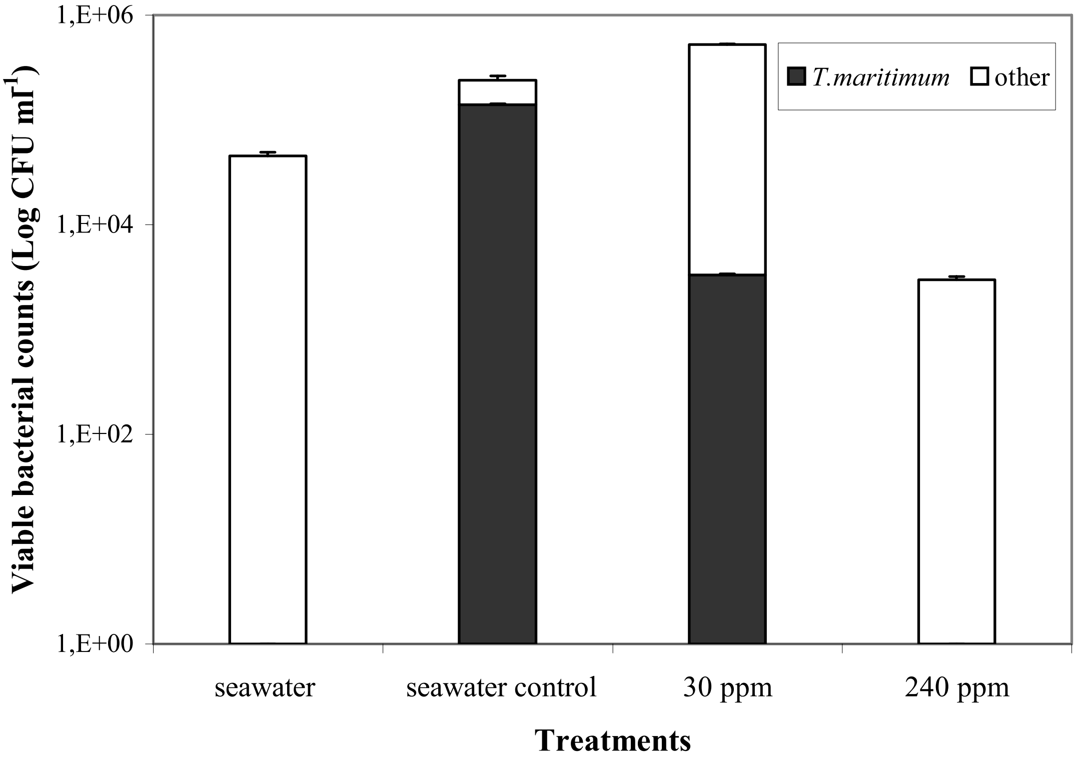


Fig. 1. Avendaño-Herrera et al.

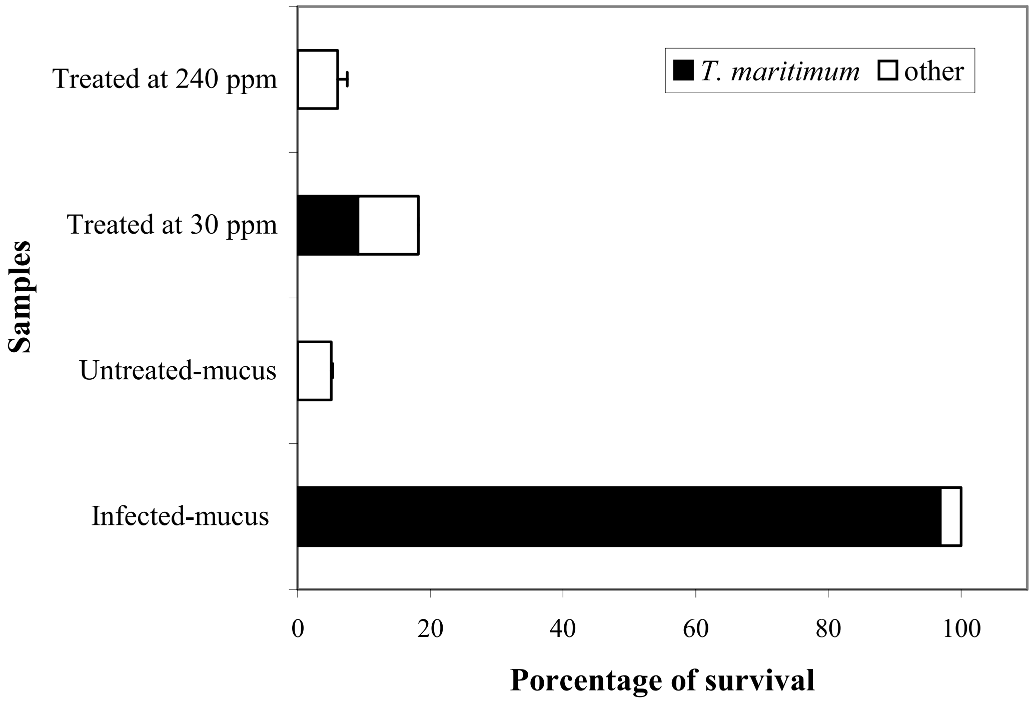


Fig. 2. Avendaño-Herrera et al.

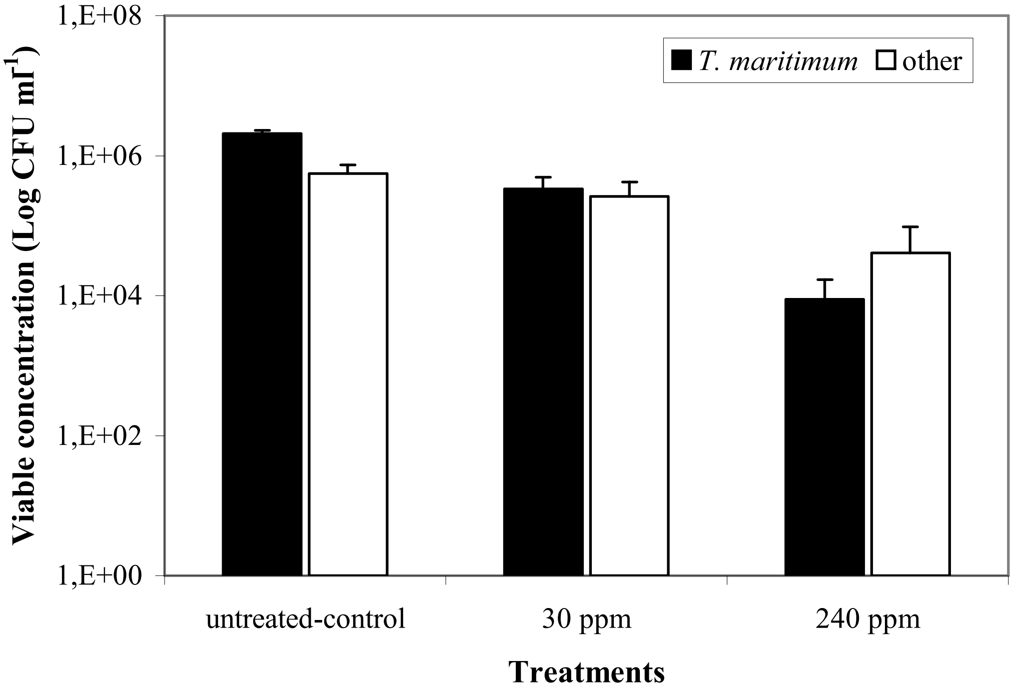


Fig. 3. Avendaño-Herrera et al.

VIII.B. LÁMINAS COMPLEMENTARIAS A LAS PUBLICACIONES

Lámina 1. Lectura de la sensibilidad a diferente agentes quimioterápicos usando los medios de cultivos *Flexibacter maritimus* (A); Anacker y Ordal (B); Agar Marino (C) y Mueller-Hinton (D) agar.

Lámina 2. Método del E-test: colocación de las tiras impregnadas con diferentes concentraciones de quimioterápicos en placas con agar *Flexibacter maritimus* de 150 mm de diámetro.

Lámina 1.

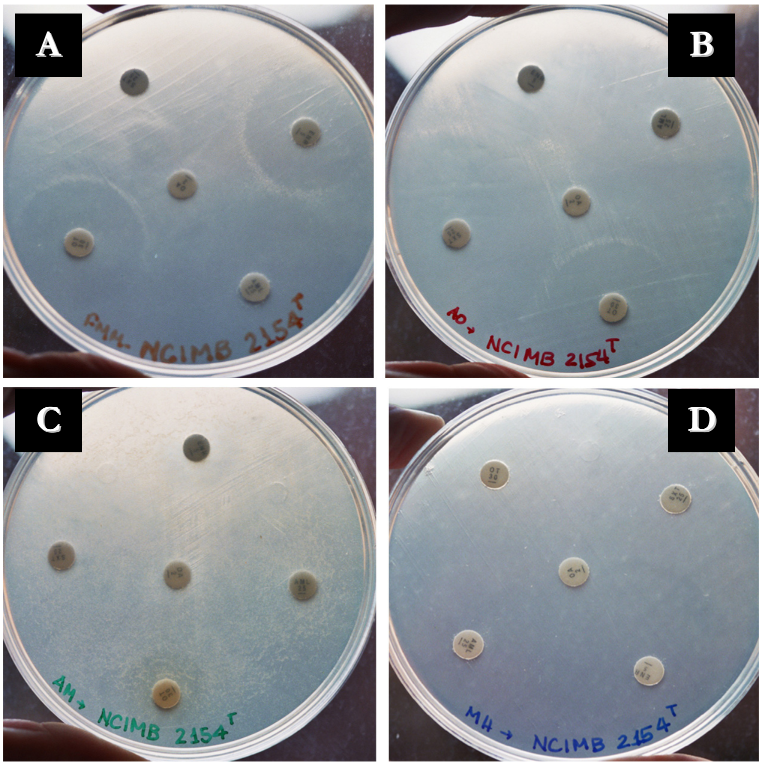
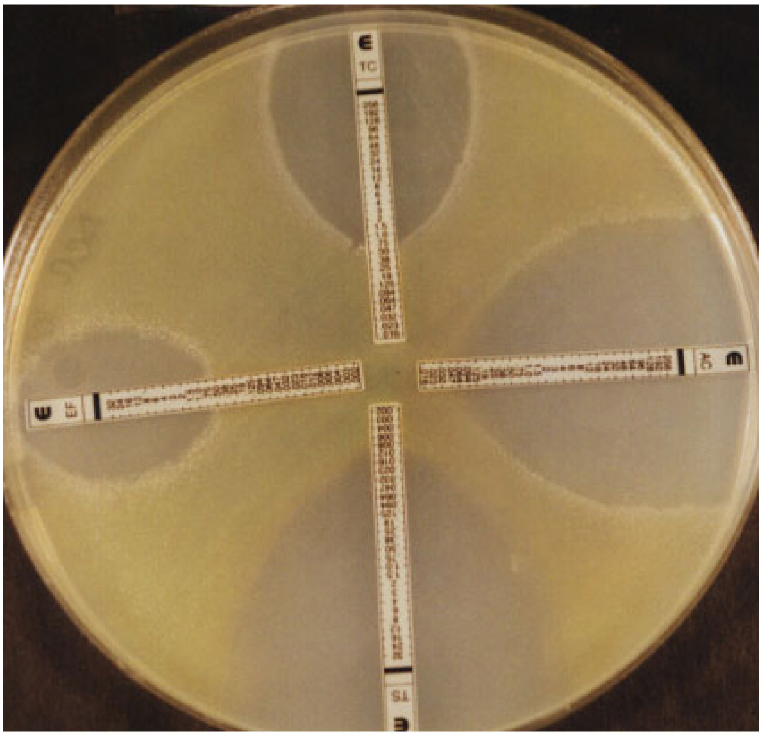


Lámina 2.



IX. DISCUSIÓN GENERAL

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Tenacibaculum maritimum, el agente causal de la tenacibaculosis en agua de mar, ha sido aislado a partir de diferentes especies de peces en distintas áreas geográficas. Como se ha expuesto en la revisión bibliográfica de la Introducción, el patógeno es responsable de importantes pérdidas económicas en granjas de cultivo de peces como rodaballo, lenguado, dorada, salmónidos y lubina en todas las fases del ciclo de crecimiento. En este trabajo se ha llevado a cabo la caracterización bioquímica, serológica y molecular de cepas de *T. maritimum* aisladas a partir de cada uno de estos huéspedes, las cuales han sido asociadas a mortalidades ocurridas en diversas piscifactorías.

La caracterización fenotípica de éstos aislados, tanto mediante métodos convencionales como utilizando el sistema API ZYM, puso de manifiesto la existencia de una gran homogeneidad en los resultados de las pruebas morfológicas, fisiológicas y bioquímicas, no encontrándose mayores diferencias entre las cepas causantes de epizootias en una determinada zona y las de procedentes de otras áreas geográficas (Avendaño-Herrera y col. 2004: artículo nº 1). Dicha homogeneidad fenotípica entre los aislados incluídos dentro de la especie *T. maritimum* había sido también descrita por otros autores (Pazos y col. 1993; Chen y col. 1995; Bernardet y Grimont 1989; Bernardet y col. 1990; Ostland y col. 1999). Además, algunos autores (Wakabayashi y col. 1986; Chen y col. 1995) citan la posibilidad de encontrar resultados variables entre las cepas para las reacciones de reducción de nitratos y producción de H_2S, las cuales también han sido observadas en el presente estudio.

A pesar de esta uniformidad a nivel bioquímico, las técnicas clásicas muestran una limitada utilidad a nivel epidemiológico puesto que no permiten establecer diferencias entre las cepas de *T. maritimum* procedentes de un mismo origen geográfico o huésped. Por ello, el conocimiento a nivel antigénico del patógeno, es un aspecto imprescindible tanto para el diagnóstico de la enfermedad, como para la selección de las cepas que permitan formular correctamente una vacuna contra la enfermedad (Romalde y col. 2005). Hasta ahora, los pocos estudios serológicos realizados en *T. maritimum* establecen la existencia de heterogeneidad antigénica dentro de la especie (Pazos 1997; Ostland y col. 1999). Sin embargo, persiste una falta de concordancia acerca de los grupos serológicos descritos y de los métodos utilizados para la caracterización antigénica del patógeno.

Considerando esta premisa, preparamos tres antisueros utilizando los antígenos "O" de dos cepas de lenguado (PC503.1 y ACC13.1) y otra de rodaballo (PC424.1) (Avendaño-Herrera y col. 2004; 2005: artículos nº 1 y 2). Los resultados de los ensayos de aglutinación en portaobjetos usando los antisueros no absorbidos mostraron reacción positiva con los antígenos "O" de todas las cepas estudiadas, independiente del huésped de aislamiento y del suero empleado. Wakabayashi y col. (1984) usando también la técnica de aglutinación en portaobjetos observaron reacciones cruzadas entre diferentes cepas de *T. maritimum* aisladas de besugo japonés *(A. schlegeli)* y pargo japonés *(P. major)*, demostrando que las cepas de *T. maritimum* comparten uno o varios antígenos y establecieron la existencia de homogeneidad antigénica para la especie *T. maritimum*.

Sin embargo, los ensayos de "dot-blot" utilizando sueros no absorbidos revelaron la existencia de heterogeneidad a nivel antigénico. Si bien, Pazos (1997) logró establecer mediante ensayos de "dot-blot" la existencia de a lo menos cuatro grupos serológicos entre aislados de rodaballo, salmón, pargo japonés, lubina, lenguado y las tres cepas de referencia, éstas diferencias serológicas intra-específicas fueron discriminadas sólo cuando utilizó sueros absorbidos, y no pudo establecer relación entre el origen de las cepas de *T. maritimum* y el grupo serológico al que éstas pertenecían. La diferencia con nuestros resultados podría ser debido a las cepas usadas para obtener los antisueros, así como los antígenos empleados para absorber los sueros.

Con el conocimiento de que la técnica de "immunoblot" incrementa la eficacia para establecer la relación antigénica e inmunológica existente entre las cepas de una misma especie bacteriana decidimos utilizar esta técnica para confirmar los resultados serológicos obtenidos y poder establecer un claro esquema de serotipado dentro de *T. maritimum*. Los ensayos de transferencia en membranas realizados con los LPS y los antisueros PC503.1, ACC13.1 y PC424.1, demostraron claramente la existencia de 3 grupos serológicos en *T. maritimum*, sin necesidad de usar sueros absorbidos. Cada grupo presentó un perfil de LPS característico, siendo desvelado por la migración de bandas, distancia inter-banda, así como la posición del núcleo oligosacarídico "core" y que hemos denominado serotipos O1, O2 y O3. El serotipo O1 está constituído por la mayoría de los aislados de lenguado procedentes del norte de España y todas las cepas de *T. maritimum* aisladas de dorada. El serotipo O2 está compuesto por todas las cepas de *T. maritimum* aisladas de rodaballo y salmón, mientras que el serotipo O3 comprende los aislados de lenguado obtenidos de epizootias ocurridas en Portugal y sur de España. De esta forma, esta técnica confirma la existencia de heterogeneidad en la composición antigénica de los LPS de la envoltura celular dentro de la especie *T. maritimum*. Dicho conocimiento es de gran importancia para el desarrollo de futuras vacunas o mejorar los programas de prevención existentes contra la tenacibaculosis marina.

El análisis de las proteínas de membrana, tanto totales como externas, mostró que todas las cepas de *T. maritimum* estudiadas, independiente del grupo serológico y la fuente de aislamiento presentan un perfil proteico similar, con masas moleculares comprendidas en un rango entre 14,2 y 66 kDa. Resultados similares han sido descritos por Pazos (1997), quien observó en los aislados de *T. maritimum* una gran cantidad de proteínas, con masas moleculares comprendidas entre 14,4 y 97,4 kDa. Sin embargo, pese a esta similitud proteica, el autor se contradice definiendo *T. maritimum* como una especie heterogénea (Pazos 1997). Por otro lado, nuestros resultados concuerdan también con el estudio de

Bernardet y col. (1994), quienes definen a *T. maritimum* como una especie homogénea en cuanto a la composición proteica de la envoltura celular. Además, el análisis de "immunoblot" ha logrado demostrar que las proteínas de los distintos aislados de *T. maritimum* están antigénicamente relacionadas, mostrando una fuerte reacción antigénica con los antisueros no absorbidos de rodaballo y lenguado.

En este trabajo hemos determinado la existencia de variabilidad genética intra-especifica entre cepas de *T. maritimum* aisladas a partir de diferentes especies de peces marinos usando la técnica de amplificación aleatoria del ADN (Avendaño-Herrera y col. 2004: artículo nº 3). Si bien, sólo tres de los seis cebadores testados (P2, P4 y P6) generaron un patrón apropiado de los productos amplificados, el análisis de éstos perfiles reveló la existencia de diferentes grupos genéticos, independientemente del cebador empleado. Al utilizar los cebadores P2 y P6, todas las cepas se separaron en dos grupos mayoritarios, fuertemente correlacionados con el huésped y/o O-serotipos descritos para este patógeno (Avendaño-Herrera y col. 2004; 2005: artículo nº 1 y 2). El primer grupo está formado por todos los aislados de lenguado y dorada, mientras que el otro grupo está compuesto por los aislados de seriola, salmón del Atlántico y rodaballo. La asignación de las cepas de referencia a los diferentes grupos genéticos varió en función del cebador utilizado. El índice de similitud estimado por el Coeficiente de Dice (S_d) entre los dos grupos fue de 32 y 75% (P2 y P6 respectivamente). Por otro lado, el cebador P4 mostró perfiles de RAPD cepa-específicos, lo cual podría ser útil para el seguimiento de clones bacterianos generadores de epizootias. Si bien, Pazos (1997) al aplicar la técnica de ribotipado demostró la existencia de diversidad intra-específica a nivel genómico dentro de la especie *T. maritimum*, los patrones de ribotipado obtenidos dependerían directamente de la enzima seleccionada para la digestión del ADN y no pueden ser utilizados con fines epidemiológicos para la determinación del origen de los aislados. Por ello, podemos concluir que el empleo de la técnica de amplificación aleatoria del ADN es una herramienta valiosa para estudios epidemiológicos de *T. maritimum*.

Otro aspecto abordado en la presente memoria ha sido evaluar la especificidad y sensibilidad de los protocolos de PCR descritos para la identificación de *T. maritimum* (Avendaño-Herrera y col. 2004: artículo nº 4). En este sentido, los resultados demuestran que el protocolo de Toyama y col. (1996) presenta una especificidad y sensibilidad mayor que el protocolo de Bader y Shotts (1998), ya que los cebadores diseñados por este último grupo de investigación no amplifican algunas cepas aisladas de lenguado. Sin embargo, los estudios de restricción llevados a cabo con las endonucleasas *Pvu*I y *Sac*II de los productos de amplificación obtenidos con los cebadores de Toyama y col. (1996), incluyendo los correspondientes a los aislados no reconocidos como *T. maritimum* por los cebadores Mar1-Mar2 (Bader y Shotts 1998), demostraron que todos los perfiles de restricción fueron idénticos. Estos resultados confirman que las cepas analizadas corresponden a *T. maritimum*. Además, los cebadores diseñados por Toyama y col. (1996) mostraron una mayor sensibilidad detectando concentraciones de 10^4 a 10^5 UFC ml^{-1} (80 a 500 células de *T. maritimum*/reacción).

En base a estos datos se desarrolló un protocolo de PCR anidada y se evaluó su eficacia en el diagnóstico de la tenacibaculosis a partir de tejidos de peces infectados (mucus, piel, riñón y sangre). Con este método se obtuvo una sensibilidad entre 1 a 250 células de *T. maritimum*/reacción (dependiendo del tejido), siendo el mucus el tejido de elección (Avendaño-Herrera y col. 2004: artículo nº 4). Teniendo en cuenta este resultado, nos planteamos la posibilidad de detectar *T. maritimum* en muestras no destructivas con el fin de conseguir un protocolo de identificación del patógeno especifico y no letal. De esta forma, analizamos 60 muestras de mucus procedentes de dorada, lenguado y rodaballo cultivados en piscifactorías españolas, detectando *T. maritimum* en el 100% de los peces con síntomas externos de la infección y en 15 de los 29 peces asintomáticos (Avendaño-Herrera y col. 2004: artículo nº 5).

Considerando los resultados obtenidos podemos señalar que el protocolo no letal desarrollado en base a la técnica de la PCR anidada posibilita la rápida detección de *T. maritimum* tanto en peces sintomáticos como portadores de la enfermedad, siendo altamente sensible, específico y de fácil aplicación para el diagnóstico y por lo tanto el control de la tenacibaculosis.

Los estudios de supervivencia realizados en microcosmos experimentales pusieron de manifiesto que *T. maritimum* sobrevive por un largo tiempo en los microcosmos con agua de mar estéril y las células permanecen cultivables después de 160 días de incubación, con concentraciones constantes de 10^3 UFC ml^{-1} y 10^6 células ml^{-1} (Avendaño-Herrera y col. 2005: artículo nº 6). Sin embargo, en los microcosmos no estériles se detectó una drástica reducción en el número de células cultivables durante los primeros 5 días post-inoculación, perdiendo la cultivabilidad tras este período. Esta falta de crecimiento en placas de agar estuvo acompañada de una reducción en el número de células, siendo demostrada cuando utilizamos el protocolo de PCR anidada, la que logró detectar la presencia de *T. maritimum* en agua natural sólo hasta el día 12 de incubación. Además, en ambos microcosmos se detectaron diferencias en la morfología y tamaño de las células de *T. maritimum*, pero no en las características bioquímicas, serológicas y genéticas.

La pérdida de cultivabilidad en microcosmos de agua de mar natural no es exclusiva de *T. maritimum* y ha sido observada también en otros patógenos bacterianos como *Aeromonas hydrophila* y *Vibrio vulnificus* (Maalej y col. 2004; Marco-Noales y col. 2004). Es bien conocido que en la mayoría de los microorganismos la pérdida de cultivabilidad va acompañada de una disminución del tamaño celular, siendo considerado este tipo de comportamiento una estrategia de supervivencia que le permiten a las bacterias minimizar los requerimientos nutricionales cuando las condiciones de manutención son adversas o inadecuadas para la misma (Colwell y Grimes 2000).

Sin embargo, los intentos de reactivación mediante la adición de nutrientes a diferentes intervalos de tiempo, no lograron la recuperación de la cultivabilidad de *T. maritimum,* indicando que, al contrario de lo que sucede en *A. hydrophila* y *V. vulnificus* (Maalej y col. 2004; Marco-Noales y col. 2004), *T. maritimum* no presenta capacidad de "reactivación". Estos resultados concuerdan con los obtenidos por Masuda y col. (2004) en sus estudios de "reactivación" de una cepa de *Tenacibaculum* sp., la cual ha sido designada recientemente como *Flexibacter echinicida* (Masuda comunicación personal).

Otra alternativa que podría explicar la reducción de la cultivabilidad de *T. maritimum* es que las células fueran inhibidas por la población bacteriana presente en el agua de mar, pudiendo afectar a la integridad celular del patógeno. Al analizar si en la microbiota nativa del agua de mar había bacterias con capacidad de producir metabolitos que resultan antagónicos para *T. maritimum*, se encontró que el 75% de las cepas aisladas, las cuales pertenecen a los géneros *Vibrio*, *Alteromonas* y *Aeromonas* presentaron actividad inhibitoria.

Todos los resultados obtenidos sugieren que *T. maritimum* es afectado drásticamente por la microbiota autóctona del agua de mar, por lo que éste medio no sería un importante vector para la transmisión de la tenacibaculosis. De todas maneras, no se puede descartar que esta bacteria pueda permanecer un cierto tiempo en el medio acuático en una concentración inferior a los límites de detección obtenidos con los métodos utilizados en nuestro estudio y que además necesite algún factor de crecimiento aún no determinado.

El siguiente paso de este trabajo fue evaluar si *T. maritimum* posee sistemas de captación de hierro, los cuales son imprescindibles para captar de los fluídos corporales de los hospedadores el hierro que necesitan para el desarrollo de la infección y que se encuentra unido a quelantes como la transferrina o lactoferrina, o bien formando parte de moléculas como la ferritina, hemosiderina, mioglobina y hemoglobina (Otto y col. 1992; Ratledge y Dover. 2000). Estos sistemas han sido estudiados en algunos patógenos de peces (Wolf y Crosa 1986; Muiño y col. 2001; Ishiguro y col. 1986; Hirst y col. 1991; Magariños y col. 1994; do Vale y col. 2002; Iida y Wakabayashi 1990; Igarashi y col. 2002; Møller y col. 2005), siendo en la mayoría de los casos asociados directamente con su virulencia.

El estudio de la existencia de mecanismo(s) de adquisición de hierro en *T. maritimum* reveló por primera vez que, independiente del grupo serológico y genético, todas las cepas de *T. maritimum* poseen algún mecanismo efectivo para la captación de este nutriente (Avendaño-Herrera y col. 2005: artículo nº 7). Así, el método químico de Schwyn y Neilands (1987), tanto en placa como en tubo, confirmó que las cepas de *T. maritimum* crecidas en medios con limitación de hierro producen sideróforos. Sin embargo, los ensayos colorimétricos de Arnow (1937) y Csáky (1948) modificado por Andrus y col. (1983) demostraron que los sideróforos sintetizados no eran compuestos de tipo fenolato o hidroxamato. La expresión de un sistema de transporte mediado por un sideróforo diferente a los convencionales ha sido descrito también en otras bacterias (Smith y col. 1985; Hu y col. 1986), lo que sugiere la necesidad de mayores estudios que permitan dilucidar la estructura química del sideróforo producido por *T. maritimum*. En cualquier caso, las sustancias transportadoras de hierro producidas por *T. maritimum* deben ser similares a nivel de especie como confirmaron los ensayos de alimentación cruzada realizados entre las cepas estudiadas.

Dado que en el interior del hospedador, el hierro es parte constitutiva de proteínas como hemina y hemoglobina, se evaluó la capacidad de las cepas para utilizar estas proteínas como fuentes de hierro, así como de otras fuentes como transferrina humana y citrato férrico amónico. Además, se valoró el efecto de la hemina y hemoglobina en la tasa de crecimiento del patógeno. Los resultados demuestran que todas las fuentes de hierro ensayadas promueven el crecimiento de los diferentes aislados de *T. maritimum* en medios restrictivos en este elemento. Además, la cinética de crecimiento del patógeno es significativamente favorecida en presencia de hemina y hemoglobina, lo que indica que el patógeno utiliza eficientemente el hierro contenido en estas proteínas como también ha sido descrito para otros microorganismos patógenos (Osorio y Lemos 2002).

Otro componente primordial en los sistemas de captación de hierro, es la presencia de proteínas u otros componentes asociados a la superficie celular que participarían como receptores en la adquisición de hierro. Independiente de los métodos probados, ninguno de aislados de *T. maritimum* fueron capaces de incrementar los niveles de absorción de hemina y rojo Congo, tanto en medios artificiales con condiciones limitantes de hierro como en medios enriquecidos, lo que sugiere la existencia de moléculas constitutivas localizadas en la superficie celular de *T. maritimum*. Esta hipótesis se corroboró al realizar el ensayo de "dot-blot" puesto que se produjo la absorción de hemina por las células completas de todas las cepas de *T. maritimum* analizadas, independiente de las condiciones de cultivo y el tratamiento previo con proteinasa-K, lo que también indica que las cepas poseen algún receptor de membrana resistente a la enzima. Resultados similares han sido obtenidos para otras especies de bacterias Gram negativas (Grenier y col. 1997; do Vale y col. 2002), en los cuales otros componentes como polisacáridos capsulares y/o LPS favorecen la absorción de hemina. En efecto, esta propiedad también fue observada cuando se empleó la fracción purificada de los LPS de *T. maritimum*.

En cuanto a la inducción de proteínas de membrana en condiciones restrictivas de hierro, al igual que ocurre en otros patógenos de peces como *L. anguillarum* (Crosa y Hodges 1981; Lemos y col. 1991; Muiño y col. 2001), *Y. ruckeri* (Romalde y col. 1991), *Ph. damselae* subsp. *piscicida* (Magariños y col. 1994) y *E. tarda* (Igarashi y col. 2002); *T. maritimum* presentó al menos tres proteínas ubicadas en la superficie externa de la célula que son reguladas por la concentración de hierro existente en el medio, de masas moleculares de 128, 85 y 66 kDa. Aunque hasta ahora se desconoce la función de estas proteínas, pero podemos especular que, como sucede en otros patógenos, algunas de ellas podrían actuar como receptoras de membrana de los sideróforos o de otros componentes de los mecanismos alternativos de adquisición de hierro.

Los resultados "*in vitro*", indican que *T. maritimum* posee sistemas de captación de hierro mediados y no mediados por sideróforos, los cuales podrían participar en el proceso infeccioso de la bacteria, así como en la proliferación de la misma en el ambiente interno del hospedador después que la bacteria patógena supera los mecanismos de defensa inespecíficos presentes en el mucus del pez (Magariños y col. 1995). Los ensayos "*in vivo*" confirmaron la hipótesis descrita anteriormente, ya que la administración de hierro a los peces, en forma de hemina, incrementó considerablemente la susceptibilidad de los peces a la infección (Ver apartado 7.1).

Hasta ahora en *T. maritimum* no se habían realizado prácticamente estudios encaminados a determinar algunas características estructurales y moleculares como la presencia de envoltura capsular y contenido plasmídico, las cuales podrían estar implicadas en la virulencia de la bacteria, al igual que ocurre en otros patógenos de peces (Toranzo y Barja 1993; Toranzo y col. 2005). Los estudios de detección de material capsular llevados a cabo con las técnicas de microscopía óptica y microscopía de transmisión, nos han permitido observar la presencia de envoltura capsular en todas las cepas estudiadas, independiente de su origen y grupo serológico. Por lo cual, no podemos descartar la posibilidad de que éste componente celular presente un potencial patogénico para los peces para lo cual son necesarios mayores estudios.

En cuanto al análisis del contenido plasmídico usando dos diferentes métodos de extracción, ninguno de ellos permitieron detectar la existencia de material extracromosomal en los aislados de *T. maritimum* incluídos en este estudio. De esta forma, no es posible establecer una correlación entre la presencia de un plásmido concreto y determinadas propiedades de virulencia asociadas al mecanismo de captación de hierro como ha sido demostrado en *L. anguillarum* (Tolmasky y col. 1985; Conchas y col. 1991; Toranzo y Barja 1993).

Como ya se indicó en el apartado de tratamiento y prevención de la tenacibaculosis (ver apartado 1.5) cuando ocurren casos de infecciones, los piscicultores comúnmente emplean agentes quimioterápicos administrados por inmersión o baños para el control de la enfermedad. Sin embargo, es un hecho bien conocido que la recuperación del patógeno desde los peces enfermos no siempre es fácil, ya que la bacteria tiene un crecimiento difícil requiriendo de medios de cultivos bajos en nutrientes y preparados con agua de mar.

Dado que la "National Committee for Clinical Laboratory Standards" (NCCLS 2003) (ahora "Clinical Laboratory Standards Institute, CLSI") recomienda una versión diluída de Mueller-Hinton para los ensayos de sensibilidad a los agentes antimicrobianos cuando se utiliza el método de difusión en placas, la primera parte del Capítulo VIII se ha centrado en determinar el medio de cultivo más adecuado para dicho fin (Avendaño-Herrera y col. 2005: artículo nº 8). Para ello, comparamos los medios de cultivos Anacker y Ordal (AOA), Agar Marino (AM) y *Flexibacter maritimus* (FMM) con la versión diluída de Mueller-Hinton (DMHA) con cinco diferentes agentes antimicrobianos. Nuestros resultados mostraron que, como cabría de esperar, ninguna cepa creció en DMHA. Es un hecho conocido que *T. maritimum* es un microorganismo estrictamente marino y los medios de cultivos requieren al menos 30% de agua de mar para permitir su crecimiento (Wakabayashi y col. 1986), así como que la bacteria presenta requerimientos de sales inorgánicas y de una baja concentración de nutrientes específicos (Pazos y col. 1996).

Aunque los ensayos de susceptibilidad a los agentes antimicrobianos usando AOA, AM y FMM demostraron que las cepas de *T. maritimum* presentan un patrón similar de susceptibilidad, siendo resistentes a ácido oxolínico y susceptibles a amoxicilina y trimetoprin sulfametoxazol, enrofloxacina y oxitetraciclina, sólo el FMM proporcionó zonas de inhibición claras, bien definidas con un rápido crecimiento para todas las cepas después de 24 h de incubación, tanto en los ensayos de difusión en placa como en E-test. Además, este medio de cultivo preparado con sales marinas comerciales (Sea Salts), en vez de agua de mar, es también apropiado para el aislamiento y su uso en ensayos de susceptibilidad de *T. maritimum*.

En conclusión, todos los ensayos indicaron que el FMM no es sólo el medio más efectivo para el aislamiento y recuperación del patógeno a partir de peces enfermos, sino también para la realización de los ensayos "*in vitro*" de susceptibilidad a los agentes antimicrobianos.

Sin embargo, a pesar de que muchas cepas de *T. maritimum* son "*in vitro*" sensibles a una gran cantidad de compuestos antibacterianos, el control de la enfermedad es difícil, debido a la posible aparición de cepas bacterianas resistentes a dichos compuestos como ha sido demostrado en otros patógenos de peces (Smith y col. 1994; Alderman y Hastings 1998). Así, Pazos (1997) observó en rodaballos cultivados en el área de Galicia que la tenacibaculosis remitía tras el tratamiento con tetraciclinas, sulfamidas potenciadas y nitrofuranos, pero aparecían nuevos brotes unos 15 días de haber finalizado el tratamiento.

A partir del año 2000, el quimioterápico más frecuentemente usado en la gran mayoría de las piscifactorías de la península Ibérica ha sido la enrofloxacina, una quinolona fluorada de nueva generación, la que inicialmente actúa en forma efectiva en el control de la enfermedad. Sin embargo, como hemos demostrado en esta memoria el uso rutinario de este quimioterápico en una piscifactoría ha dado lugar a la aparición de cepas de *T. maritimum* resistentes a dicho compuesto, con la siguiente dificultad en el control de la tenacibaculosis (Avendaño-Herrera y col. 2005: artículo nº 9). Estudios encaminados a determinar la CMI de este compuesto mediante la técnica de micro-dilución en placas mostraron un efecto bactericida importante frente a las cepas de *T. maritimum* (CMIs 80% entre 0,5 a 32 μg ml^{-1}). Los análisis de agrupamiento pusieron de manifiesto la existencia de 2 grupos diferentes, constituídos por las cepas consideradas como susceptibles con una CMI promedio de 1,1 μg ml^{-1} y aquellos aislados con valores de CMI superiores a 13,5 μg ml^{-1}, siendo considerados como resistentes a la enrofloxacina. Es importante señalar que los valores de inhibición de *T. maritimum* cuando se utiliza dicho compuesto fueron significativamente superiores a los descritos en otros patógenos bacterianos de peces como *Y. ruckeri* (0,005 μg ml^{-1}), *L. anguillarum* (0,032 μg ml^{-1}) y *A. salmonicida* (0,012 μg ml^{-1}) (Dalsgaard y Bjerregaard 1991). Estos valores concuerdan con los señalados por Intorre y col. (2000), quienes sugieren que la mayoría de patógenos de peces tienen valores de CMI de aproximadamente 0,16 μg ml^{-1}.

Este problema medioambiental de la facilidad de generación de resistencias y el hecho de que la legislación Europea es cada vez más restrictiva al uso de antibacterianos en la producción de especies con destino a la alimentación humana han generado la prohibición de varios compuestos que habían sido recomendados y utilizados contra *T. maritimum* tales como los nitrofuranos y el cloranfenicol. En este sentido, solamente está legalmente permitido la utilización de aquellos agentes quimioterápicos que presenten niveles mínimos de residuos (MRL) y estén expresamente autorizados para peces. Por ello, en la actualidad existen muy pocos compuestos que tengan licencia, aunque sea provisional para poder ser legalmente aplicable a peces.

Todos estos inconvenientes son un factor decisivo en la potenciación de estudios encaminados a la búsqueda de métodos alternativos para el control de la tenacibaculosis. Los desinfectantes como el peróxido de hidrógeno (H_2O_2), aparte de su uso en profilaxis, han recibido una gran atención por su efectivo control de diversos patógenos externos de peces, particularmente aquellos del género *Flavobacterium* y *Cytophaga* (Lumsden y col. 1998; Derksen y col. 1999; Thomas-Jinu y Goodwin, 2004). Sin embargo, hasta el momento no se habían realizado estudios con el fin de conocer el efecto del H_2O_2 sobre el crecimiento de *T. maritimum*, así como las posibles consecuencias en los peces tratados.

En esta memoria hemos demostrado que las concentraciones de H_2O_2 ensayadas "*in vitro*" afectan drásticamente al crecimiento del patógeno de peces *T. maritimum* tras 30 minutos de incubación (Avendaño-Herrera y col. 2005: artículo nº 10). Los intentos de reactivación mediante la adición de nutrientes a las 24 h, no lograron la recuperación de la cultivabilidad de *T. maritimum* en agua de mar estéril. Sin embargo, cuando se ensayó la recuperación de esta bacteria en agua de mar natural tratada con 30 ppm, se observó un débil incremento de la viabilidad de *T. maritimum* con concentraciones alrededor de 10^3 UFC ml^{-1}. Por otro lado, la microbiota nativa del agua de mar muestra una alta supervivencia, en todas las condiciones ensayadas. Probablemente, la resistencia bacteriana es provocada por la mayor acción de enzimas antioxidantes como la superóxido dismutasa y catalasa, las cuales descomponen más rápidamente los 30 ppm a radicales de peróxido. Recientemente, Díaz-Rosales y col. (2003) han demostrado esta actividad enzimática en el patógeno de peces *Ph. damselae* subsp. *piscicida*.

Cuando analizamos la posibilidad de utilizar H_2O_2 como medida profiláctica en el agua de los peces infectados con *T. maritimum*, se obtuvo una disminución de la cultivabilidad de *T. maritimum* entre 10^2 a 10^3 UFC ml^{-1} dependiendo de la concentración de desinfectante empleada. Se sabe que la capa externa mucosa de los peces constituye una entrada de la bacteria, ya que este patógeno posee una gran capacidad de adherencia al mucus de los peces y resistencia a la acción bactericida de este material mucoso (Magariños y col. 1995). Por tanto, la bacteria puede permanecer un mayor tiempo en el medio acuático usando como reservorio el mucus de los peces. Durante el tratamiento con el desinfectante se observó un incremento en actividad respiratoria, movimiento opercular y natación circular de los diferentes grupos de peces tratados con respecto al control. Esta actividad es importante, pues aumenta la susceptibilidad de los peces a *T. maritimum* y a otros patógenos oportunistas causando la mortalidad del 100% de los peces utilizados en este estudio. De igual modo, mayores niveles de H_2O_2 (480 ppm) fueron altamente tóxicos para los peces. En este sentido, numerosos estudios señalan que la toxicidad depende de otros factores, entre ellos, la temperatura del agua, número y duración de los tratamientos, edad mínima y tamaño del pez y condiciones del agua de cultivo (Rach y col. 1997; Lumsden y col. 1998; Gaikowski y col. 1999; Derksen y col. 1999; Thomas-Jinu y Goodwin 2004).

Por tanto, podemos concluir que aunque *T. maritimum* es susceptible al tratamiento con H_2O_2 en el agua de mar cuando se emplea 240 ppm del desinfectante, el patógeno puede resistir a la acción bactericida de este compuesto causando mortalidad. De esta forma, nosotros recomendamos el H_2O_2 sólo como método general de desinfección del agua de cultivo y la superficie de los tanques antes de la introducción de los peces. Serán necesarios estudios más amplios para determinar la influencia del tamaño del pez en la respuesta al tratamiento del H_2O_2.

X. CONCLUSIONES

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A partir de los resultados obtenidos a lo largo de esta memoria podemos concluir:

1. La caracterización serológica demostró que *Tenacibaculum maritimum* es una especie antigénicamente heterogénea, principalmente en base a las diferencias en la cadena "O" de los LPS. El análisis del patrón de los LPS usando sueros no absorbidos, mostró la existencia de tres serotipos mayoritarios en función de la cadena "O", los cuales están estrechamente asociados al hospedador.

2. La técnica de amplificación aleatoria del ADN confirma la existencia de variabilidad genética intra-especifica dentro de *Tenacibaculum maritimum*, constituyéndose en una técnica molecular valiosa para estudios epidemiológicos de este patógeno. Así, se han descrito dos líneas clonales mayoritarias, las cuales están estrechamente asociadas al huésped de aislamiento.

3. De los protocolos de PCR descritos para la identificación de *Tenacibaculum maritimum*, el protocolo de Toyama y col. presenta una especificidad y sensibilidad mayor que el protocolo de Bader y Shotts. Además, proponemos el protocolo no letal basado en la técnica de PCR anidada desarrollado en nuestro estudio, para la detección rápida de *Tenacibaculum maritimum* (7 horas) tanto en peces sintomáticos como portadores de la enfermedad, siendo un método altamente sensible, específico y de fácil aplicación.

4. Los experimentos de supervivencia de *Tenacibaculum maritimum* en medio acuático pusieron de manifiesto que este patógeno es afectado drásticamente por la microbiota autóctona del agua de mar, por lo que el agua de los tanques de cultivo no sería un importante vector para la transmisión de la tenacibaculosis.

5. Todos los aislados de *Tenacibaculum maritimum*, independientes del grupo serológico poseen un sistema de alta afinidad por el hierro mediado por sideróforos, de naturaleza distinta a compuestos de tipo fenolato o hidroxamato, y un sistema alternativo, no mediado por sideróforos, que le permite utilizar el hierro unido a compuestos de tipo hemo. Al menos algunos de estos sistemas funcionan "*in vivo*" ya que la administración a los peces de hemina, previamente a la inoculación del patógeno, produjo un aumento significativo de su grado de virulencia.

6. Dentro de los estudios encaminados al posible control y prevención de la enfermedad podemos concluir que:

    a. El medio de cultivo FMM es el más adecuado para determinar la susceptibilidad "*in vitro*" de *Tenacibaculum maritimum* a los diferentes agentes antimicrobianos usados en acuicultura, por lo cual recomendamos su inclusión en una futura revisión de la norma NCCLS M42.

    b. El uso rutinario del quimioterápico enrofloxacina en plantas de cultivo causa una rápida aparición de cepas de *Tenacibaculum maritimum* resistentes a dicho compuesto, la cual no está asociado a la existencia de contenido plasmídico.

    c. La desinfección del agua de cultivo y la superficie de los tanques con peróxido de hidrógeno en una concentración de 240 ppm antes de la introducción de los peces, constituye un método alternativo para prevenir la aparición de brotes de tenacibaculosis.

XI. BIBLIOGRAFÍA

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