**Departamento de Microbiología y Parasitología**

**Facultad de Biología**

# Descripción de tres nuevas especies del Género *Tenacibaculum* causantes de tenacibaculosis: aspectos taxonómicos y patogenicidad

**Maximino Piñeiro Vidal**

2008

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**Universidad de Santiago de Compostela**

# Descripción de tres nuevas especies del Género *Tenacibaculum* causantes de tenacibaculosis: aspectos taxonómicos y patogenicidad

Memoria que presenta

**MAXIMINO PIÑEIRO VIDAL**

Para optar al Grado de Doctor por la

Universidad de Santiago de Compostela

Santiago de Compostela, Julio 2008

**YSABEL SANTOS RODRIGUEZ**, **Profesor Titular del Departamento de Microbiología y Parasitología de la Universidad de Santiago de Compostela,**

**INFORMA**: Que la presente Tesis Doctoral titulada "**Descripción de tres nuevas especies del Género *Tenacibaculum* causantes de tenacibaculosis: aspectos taxonómicos y patogenicidad**" que presenta D. **MAXIMINO PIÑEIRO VIDAL** para optar al grado de Doctor por la Universidad de Santiago de Compostela, ha sido realizada en el Departamento de Microbiología y Parasitología bajo mi dirección, y considerando que se halla concluida, autorizo su presentación para que pueda ser juzgada por el tribunal correspondiente.

Director Autor:

Fdo: **Dra. Ysabel Santos Rodríguez** Fdo: **D. Maximino Piñeiro Vidal**

**El trabajo presentado en esta memoria ha sido financiado por los proyectos de la Xunta de Galicia:**

**PGIDIT 04 RMA 003E**

**PGIDIT 06 RMA 024E**

**INCITE 07PX2081174ES**

## AGRADECIMIENTOS

Quisiera de antemano que la brevedad de estas palabras no se malinterpretase creyendo que un empacho de alegría por la finalización de esta etapa, me hiciese olvidar a todas aquellas personas que me han ayudado de modo desinteresado a rematar este trabajo. Asimismo aprovecharé este pequeño espacio sobre el papel para poder dar libertad a palabras y sentimientos. Bastantes son los que me han mostrado interés por mi trabajo, bastantes los que se han quedado atrás, menos los que optaron por lo opuesto.

Sin más quisiera agradecer de modo muy especial a Ysabel su labor como tutora y compañera. La labor realizada a lo largo de estos años por la consecución de este trabajo la honra no sólo como profesional si no como persona, algo tan escaso como valioso en estos tiempos y lugares en los que nos ha tocado vivir.

A mi esposa Rocío, con la que quisiera recordar en el ocaso de nuestras vidas estos momentos. Te quiero Ro.

A mi familia, Pa, Ma, Mari y María. Ellos ya lo saben. A mi abuela Carmen. El agnosticismo que profeso no me impide saber que si soy yo el engañado, me verá algún día desde lo más alto del cielo.

A mi compañera de laboratorio estos años, Cristina, y a mis nuevas compañeras Cris y Naiara.

Asimismo quisiera también recordar ahora a toda aquella gente que de un modo u otro estuvo cuando se les necesito. Jaime Fábregas, Gonzalo Álvarez, Ángeles Muñoz, David Arahal, Manuel Romero, Ana Riaza… ha sido un placer. Gracias.

**INDICE**

Índice

# Capítulo I. Introducción

# 1. INTRODUCCIÓN

## 1.1.- La acuicultura en Galicia: interés de los estudios patológicos

Galicia es considerada como una de las mayores potencias en pesca y acuicultura a nivel mundial. El sector de la acuicultura en Galicia, merced al desarrollado cultivo del mejillón y a la franca expansión en los últimos años de los cultivos de peces marinos, se ha posicionado en vanguardia a nivel europeo y mundial.

La acuicultura, según cifras de la FAO (2003), pasó de producir 10 millones de toneladas en 1987/88 a más de 60 millones en la actualidad, estando previsto una producción de 100 millones para el año 2030. España se encuentra entre los 10 primeros productores a nivel mundial, posición que se puede mejorar si se apuesta de forma clara por el sector. Prueba de ello, es el espectacular crecimiento del cultivo de rodaballo en Galicia, desde unas pocas toneladas en la década de los 80, hasta más de 5.500 Tm por año en la actualidad.

La Comisión de la Unión Europea, consciente de este crecimiento, presentó una Comunicación al Consejo y Parlamento Europeo sobre la "Estrategia para el desarrollo sostenible de la Acuicultura Europea", en la que figura como actuaciones a llevar a cabo por este sector: i) fomentar la viabilidad económica, ii) solucionar los problemas medioambientales y iii) garantizar la seguridad de los alimentos y la salud y bienestar de los animales.

La realización de estudios patológicos es, sin duda, una de las principales vías para garantizar la rentabilidad de la industria y asegurar el bienestar de los animales en cultivo y la calidad y salubridad de los productos ofertados al consumidor.

Las empresas de acuicultura deben tener un conocimiento claro de las patologías a las que los individuos de la especie cultivada están expuestos y han de estar capacitadas para hacer frente a las infecciones de etiología bacteriana, vírica o parasitaria que puedan surgir a lo largo del ciclo de producción.

Entre las enfermedades de etiología bacteriana de mayor importancia para la piscicultura marina de nuestra área destacan la vibriosis por *Vibrio anguillarum*

(sinónimo, *Listonella anguillarum*) y otras especies del género *Vibrio*, la furunculosis producida por *Aeromonas salmonicida* subespecie *salmonicida* y la flexibacteriosis o tenacibaculosis causada por *Tenacibaculum maritimum* (sinónimo *Flexibacter maritimus*) y microrganismos relacionados taxonómicamente (Piñeiro-Vidal, 2004; Santos, 2005).

Asimismo, es de destacar la detección y/o aislamiento de otras bacterias pertenecientes a los géneros *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Staphylococcus* y *Streptococcus*, como únicos agentes causantes de infección o en infecciones mixtas asociadas con otras bacterias o parásitos.

## 1.2.- La tenacibaculosis marina

### 1.2.1.- Introducción histórica y patología

La tenacibaculosis o flexibacteriosis marina fue descrita por primera vez en alevines de pargo japonés (*Pagrus major*) y besugo japonés (*Acanthopagrus schlegeli*) cultivados en Japón (Hikida y col., 1979). La enfermedad se diagnosticó por vez primera en Europa a finales de los años 70, bajo el término de enfermedad de los puntos negros "*Black Patch Necrosis*" (BPN, McVicar y White, 1979), en cultivos de lenguado en Escocia. Sin embargo, la identificación de la bacteria filamentosa implicada, de características parecidas a *Flavobacterium columnare* (Campbell y Buswell, 1982), no se realizó hasta el año 1990 (Bernadet y col., 1990). Desde entonces la enfermedad ha sido descrita bajo diferentes denominaciones (tales como BPN, enfermedad de bacterias deslizantes en peces marinos, flexibacteriosis marina, podredumbre de aletas y cola y síndrome de la boca erosionada, enfermedad similar a la causada por *F. columnare*), que hacen referencia a los signos clínicos que se observan en los peces afectados (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, Cepeda y Santos, 2002), siendo siempre aislada la bacteria *Tenacibaculum maritimum* como el agente causal de la enfermedad. Hansen y col. (1992), describen una nueva especie dentro del género

*Flexibacter*, patógena para huevos y larvas de halibut atlántico (*Hippoglossus hippoglossus* L.) y proponen para ella el nombre específico de *Flexibacter ovolyticus* (sinónimo *Tenacibaculum ovolyticum*, Suzuki y col., 2001). Este microorganismo no se ha aislado de otros peces en cultivo a pesar de que mediante infecciones experimentales se ha demostrado que puede causar la muerte de huevos y larvas de bacalao (Bergh, 2000). Recientemente, Miguez y Combarro (2003) han descrito el aislamiento de *T. maritimum* y *T. ovolyticum* a partir de huevos de sardina (*Sardina pilchardus*) en ambientes naturales. Sin embargo, la presencia de estos microorganismo no afecta a la viabilidad de los huevos de sardina, quizás debido a que se encuentran en bajos niveles en la superficie de los mismos (Miguez y col., 2004). Actualmente, *T. maritimum* es el principal agente causante de mortalidades en la mayor parte de las especies en cultivo y en peces salvajes, mientras que *T. ovolyticum* es considerado un patógeno oportunista (Hansen y col., 1992; Bernardet, 1997, Bergh y col., 2001; Samuelsen y col., 2006). Recientemente, nuestro grupo ha aislado otras bacterias filamentosas patógenas para peces, con caracteres fenotípicos similares a *T. maritimum*, a partir de rodaballos y lenguados enfermos y del agua de mar de los tanques de cultivo. Estos microorganismos serán el objeto de estudio del presente trabajo de investigación.

Con respecto a la patología de la enfermedad, las lesiones ulcerativas sobre la superficie externa, son el signo clínico más característico de la tenacibaculosis causada por *T. maritimum* y bacterias filamentosas relacionadas. El tamaño y la localización de las lesiones difieren en función de la especie (Chen y col., 1995; Handlinger y col., 1997) y la edad del pez (Baxa, 1988). Las lesiones aparecen con mayor frecuencia sobre la superficie de la piel de las aletas, cola, opérculo o cabeza. También se han observado lesiones en las agallas en salmón chinook (*Oncorhynchus tschawytscha*) (Chen y col., 1995), en salmón atlántico (*Salmo salar*) y trucha arcoiris (*O. mykiss)* (Handlinger y col., 1997). El tejido orbital ocasionalmente se ve afectado (Handlinger y col., 1997). La Figura 1 muestra algunos de los signos externos de tenacibaculosis observados en rodaballo y lenguado.

En estadíos tempranos de la infección aparece un oscurecimiento del tejido interradial, sobre todo entre las aletas caudal y marginal, donde se muestra una consistente fragmentación y degeneración del epitelio. En este proceso se observa infiltración de material de tipo proteico, acompañado con congestión y hemorragia de la

dermis superficial. Estas lesiones se extienden a la dermis y capas musculares más profundas, causando fuertes hemorragias.

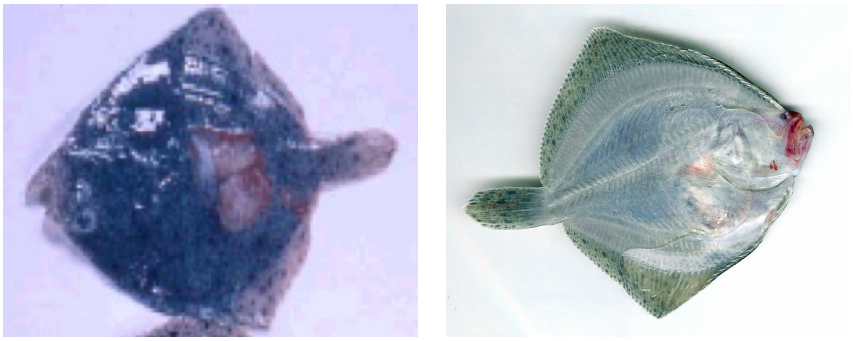
Las lesiones ulcerosas características de la enfermedad favorecen la invasión por otros agentes infecciosos (Campbell y Buswell, 1982; Chen y col., 1995; Handlinger y col., 1997; Bodammer, 2000; Law, 2001; Mastan y Qureshi, 2001; 2003; Udomkusonsri y col., 2004), lo que agrava el estado del pez. En otras ocasiones, las heridas producidas por la multiplicación de otros microorganismos patógenos o las abrasiones mecánicas van a favorecer el desarrollo de la enfermedad (Chen y col., 1995; Kimura y Kusuda, 1983).

La enfermedad puede tener finalmente un carácter sistémico y, a pesar de que internamente no se observan daños, se ha aislado el patógeno de riñón, bazo o hígado (Baxa y col., 1988; Alsina y Blanch, 1993; Pazos, 1997; Ostland y col., 1999; Santos y col., 1999; Cepeda y Santos, 2002; Avendaño-Herrera y col., 2006).

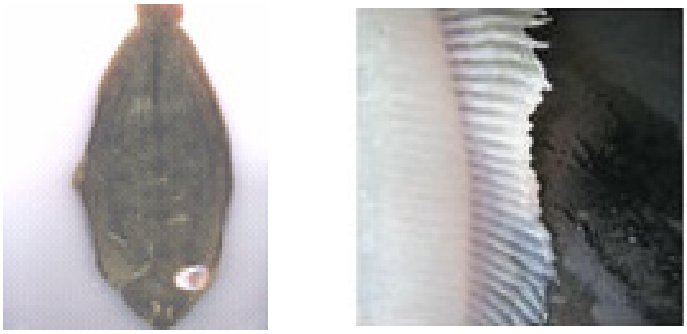
Los peces afectados muestran natación errática, inapetencia, adelgazamiento e irregularidades respiratorias (Devesa y col., 1989), debido al daño a nivel de las branquias y a la pérdida de sangre. Además, la presencia de distintos tipos de exotoxinas producidas durante la multiplicación del patógeno en los tejidos de los peces afectados (Baxa y col., 1987; Dalsgaard, 1993; Pazos, 1997), puede afectar al funcionamiento de órganos internos alterando el comportamiento natural del pez.

Histológicamente, no se han descrito cambios en órganos internos. Sí se observan, en cambio, lesiones en la piel caracterizadas por desprendimiento del epitelio de revestimiento, infiltrado inflamatorio en la dermis de leve a moderado y necrosis muscular con presencia de gran cantidad de bacterias (Failde y col., 2008). La Figura 2 muestra las lesiones producidas por *T. maritimum* en piel y musculatura de lenguado *Solea senegalensis* (imágenes reproducidas con permiso de Failde y col., 2008).

Las manifestaciones clínicas características de la tenacibaculosis causada por *T. ovolyticum* y que conllevan a la muerte de los huevos y larvas de halibut son la disolución del corion y zona subyacente y la liberación del contenido celular por acción de las exotoxinas (Hansen y col., 1992; Austin y Austin, 1999; Austin y Austin, 2007).



A



B

**Fig 1**.- Signos externos de tenacibaculosis marina en rodaballo (*Psetta maxima*) (A) y lenguado senegalés (*Solea senegalensis*) (B).

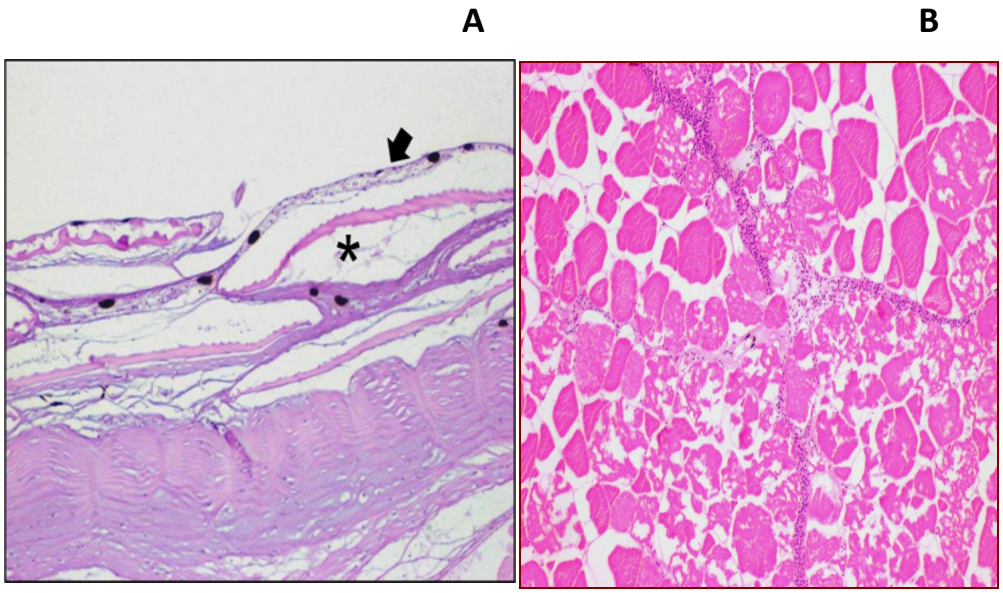



**Fig 2**.- Lesiones en peces afectados por tenacibaculosis marina. A) Pérdida de la epidermis (flecha) y dilatacion de bolsillos escamosos (asterisco). B) degeneración de los paquetes musculares rodeada por una reacción inflamatoria leve. Hematoxilina Eosina 200X.

### 1.2.2.- Transmisión y reservorio

El modo de transmisión y la vía de infección de *Tenacibaculum maritimum* y otras *Tenacibaculum* spp. patógenas de peces son todavía inciertos. Los ensayos de patogenicidad han demostrado que cuando los peces se exponen a la aplicación tópica de *T. maritimum* (Wakabayashi y col., 1984) o a una combinación de aplicación tópica e inmersión en cultivos bacterianos (Baxa y col., 1987) se producen mortalidades sensiblemente superiores a las observadas cuando las infecciones experimentales se realizaban mediante inyección intramuscular o mediante baño. Estudios de infección, por inmersión de los peces en una suspensión de la bacteria durante períodos prolongados (60-90 minutos a 18 horas), sugieren que el agua también podría ser una ruta de infección de *T. maritimum* en salmón del Atlántico, trucha arcoiris (*Oncorhynchus mykiss*), "greenback flounder" (*Rhombosolea tapirina*) y rodaballo (*Psetta maxima*) (Soltani y col., 1996; Handlinger y col., 1997; Avendaño-Herrera y col., 2006).

Los reservorios naturales del patógeno son desconocidos. *T. maritimum* fue aislado a partir de sedimentos, de agua y de la superficie de tanques que habían estado en contacto con peces infectados (Carson y col., 1993; Santos y col., 1999). Recientemente, Avendaño-Herrera y col (2006) estudiando la supervivencia de *T. maritimum* en agua de mar, demostraron que la bacteria sobrevive hasta 5 meses en agua de mar estéril pero deja de ser cultivable en tan sólo cinco días cuando se emplea agua de mar no estéril en los ensayos. Estos investigadores concluyen que el agua de mar no es una vía de transmisión de *T. maritimum*. Sin embargo, no se puede descartar que en ambientes naturales o en los sistemas de cultivo de peces, la bacteria pueda mantenerse en estado viable no cultivable en el agua, sedimento o adherida al mucus de los peces hasta que las condiciones ambientales sean favorables para el crecimiento. De hecho, se ha establecido que *T. maritimum* posee una gran capacidad de adherencia al mucus de dorada (*Sparus aurata*) y rodaballo, así como resistencia a la acción bactericida del mucus (Magariños y col., 1995; Pazos, 1997).

### 1.2.3.- Rango de hospedadores y distribución geográfica

*Tenacibaculum maritimum* se ha aislado a partir de numerosas especies y en regiones geográficas muy diversas. En un principio se describió como el agente causal de mortalidades masivas en varias especies de peces marinos en Japón (Masumura y Wakabayashi, 1977; Baxa y col., 1986). Posteriormente, este patógeno ha sido aislado en Europa, América y Australia a partir de numerosas especies de peces de interés económico como lenguado (*Solea solea* y *S. senegalensis*) (Campbell y Buswell, 1982; Cepeda y Santos, 2002), salmón atlántico (*Salmo salar*) (Schmidtke y col., 1991, Ostland y col., 1999), salmón coho (Pazos y col., 1993), salmón chinook (*O. tschawytscha*), rodaballo (*P. maxima*) (Alsina y Blanch, 1993; Pazos y col., 1993), lubina (*Dicentrarchus labrax*) (Bernardet y col.,1994), trompeta rayada (*Latris lineata*), "yellow eye-mullet" (*Aldrichetta forsteri*) y besugo negro (*Acanthopagrus butcheri*) (Soltani y Burke 1996; Handlinger y col., 1997). *T. maritimum* ha sido también identificado como agente causal de tenacibaculosis en poblaciones naturales de corvina blanca (*Atractoscion nobilis*), anchoa del Norte (*Engraulis mordax*) y sardina del Pacífico (*Sardinops saxax*) (Chen y col., 1995). *Tenacibaculum ovolyticum*, se ha aislado como patógeno sólo a partir de huevos y larvas de halibut atlántico (*Hippoglossus hippoglossus*) (Hansen y col., 1992) en el Norte de Europa. Recientemente, se ha descrito el aislamiento de *T. maritimum* y *T. ovolyticum* como parte de la biota epifita de huevos de sardina (*Sardina pilchardus*) en ambientes naturales (Miguez y Combarro, 2003; Miguez y col., 2004).

La tenacibaculosis marina ha sido, desde principios de los 90, un problema continuo en la piscicultura marina de Galicia. Desde 1992 *Tenacibaculum maritimum* ha sido el principal agente causal de mortalidades en rodaballo, siendo aislado esporádicamente a partir de salmón y dorada (Pazos y col., 1993; Pazos, 1997; Santos y col., 1999). A partir del año 2000, *T. maritimum* ha sido aislado de forma continuada a partir de lenguado cultivado en nuestra comunidad (Cepeda y Santos, 2002; Santos, 2005).

Más recientemente, se han aislado a partir de rodaballos, lenguados y lubinas enfermas y del agua de mar de los tanques de cultivo otras bacterias filamentosas relacionadas a nivel fenotípico con *Tenacibaculum maritimum* y que hemos denominado

como *T. maritimum* atípicos o bacterias relacionadas con *T. maritimum* (Piñeiro-Vidal, 2004).

### 1.2.4- Métodos de diagnóstico

#### *Métodos convencionales*

El diagnóstico presuntivo de la enfermedad se basa en la observación de los signos clínicos en combinación con el examen microscópico de preparaciones en fresco o teñidas obtenidas a partir de las lesiones. Sin embargo, sólo el aislamiento en cultivo puro de la bacteria y su posterior caracterización mediante la aplicación de las técnicas clásicas de identificación, métodos moleculares o serológicos, permiten el diagnóstico confirmativo de la enfermedad (Toyama y col., 1996; Pazos, 1997; Bader y Shotts, 1998; Santos y col., 1999; Bernardet y col., 2002; Jensen y col., 2002; Cepeda y col., 2003; Avendaño-Herrera y col., 2004a).

Para el aislamiento de las bacterias filamentosas a partir de peces o para su cultivo en laboratorio se han descrito diferentes medios. Entre ellos, los medios no selectivos Agar Anacker y Ordal (AOA) (Anacker y Ordal, 1955) modificado preparado en agua de mar al 70%, el Agar Marino (AM 2216E, Difco. EEUU) (Campbell y Buswell, 1982), el medio *Flexibacter maritimus* (FMM) (Pazos y col, 1996) y el medio *Tenacibaculum maritimum* (TM) (Cepeda, 2003) o el medio selectivo para *Flexibacter* (SFM) (Bullock y col., 1986) son los más utilizados en los laboratorios de diagnóstico. Para el cultivo en laboratorio o para el estudio de caracteres fenotípicos se han descrito 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 Caldo Luria (Suzuki y col., 2001) preparados en agua de mar artificial, pero no son utilizados rutinariamente para el aislamiento de la bacteria a partir de sistemas de cultivo de peces.

Para la identificación de los aislados se utilizan métodos convencionales basados en las características morfológicas, fisiológicas y bioquímicas del patógeno (Hikida y

col., 1979; Wakabayashi y col., 1984 y 1986; Bernardet y col., 1994; Chen y col., 1995; Handlinger y col., 1997; Ostland y col., 1999; Bernardet y col., 2002). Las galerías comerciales API ZYM y API 50CH (BioMérieux, Francia), han sido ampliamente utilizadas y ofrecen resultados en tan sólo 24 horas (Bernardet y col., 1994; Pazos, 1997; Santos y col., 1999; Bernardet y col., 2002).

***Métodos serológicos***

Para el diagnóstico de la flexibacteriosis marina se han propuesto distintas técnicas basadas en la reacción antígeno-anticuerpo (Tabla 1). La aplicación de técnicas serológicas también ha permitido la demostración de la existencia de antígenos comunes y antigenos diferenciales entre las distintas cepas de *T. maritimum* (Wakabayashi y col., 1984; Baxa y col., 1988 b; Pazos y col., 1993; Pazos, 1997; Ostland y col., 1999; Santos y col., 1999, Avendaño-Herrera y col., 2004 a). Las técnicas basadas en la aglutinación tienen un uso limitado para esta bacteria debido a la existencia de cepas autoaglutinantes. Otras técnicas serológicas descritas (Tabla 1) incluyen los ensayos Dot Blot e immunoblot, la inmunodifusión, el ensayo inmunoenzimático (ELISA) e inmunofluorescencia directa. Aunque estos métodos presentan alta sensibilidad, son costosos, lentos y requieren el aislamiento del microorganismo en cultivo puro. Los ensayos de detección de *T. maritimum* mediante técnicas de inmunofluorescencia indirecta e inmunohistoquímica aplicadas a tejidos infectados han dado resultados variables. 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. Sin embargo, Failde y col. (2008) encontraron resultados satisfactorios aplicando la inmunohistoquímica a la detección de *T. maritimum* en tejidos de lenguado senegalés infectados experimentalmente.

**Tabla 1**.- Técnicas serológicas utilizadas en la caracterización de las especies patógenas de *Tenacibaculum*.

| **Autor** | **Técnica serológica** |
|---|---|
| Wakabayashi y col.,1984 | Microaglutinación |
| | Microaglutinación |
| Baxa, 1988 | Doble inmunodifusión |
| | Inmunofluorescencia directa |
| | Aglutinación en portaobjetos |
| Pazos, 1997 | "Dot Blot" |
| Avendaño-Herrera y col., 2004 a | "Immunoblot" |
| Ostland y col., 1999 | "Immunoblot" |
| | Doble difusión |
| | Microaglutinación |
| Failde y col., 2008 | Inmunohistoquímica |
| Arenas y col., 2003 | ELISA |
| Powell y col., 2004 | Inmunofluorescencia indirecta |

***Métodos moleculares***

La aplicación de la técnica de la Reacción en Cadena de la Polimerasa (PCR) ha facilitado el diagnóstico de la tenacibaculosis marina. Toyama y col. (1996) y Bader y Shotts (1998) desarrollaron dos pares de cebadores y dos programas de amplificación para la identificación de *Tenacibaculum maritimum*, basándose en las secuencias disponibles de la región del DNA que codifica el RNA 16S en *T. maritimum* (Woese y col., 1990). Posteriormente, se han diseñado ensayos de alta sensibilidad que combinan PCR y ELISA (Wilson y col., 2002), transcripción reversa-PCR (RT-PCR) con hibridación en medio líquido (Wilson y Carson, 2003) y PCR e hibridación en medio sólido empleando como soporte una matriz que contiene las sondas específicas (PCR-DNA Microarrays) para detectar *T. maritimum* (Warsen y col., 2004). Todos estos sistemas son de utilidad para el diagnóstico confirmativo de la enfermedad, al permitir la identificación de la bacteria previamente aislada en cultivo puro. Sin embargo, no se ha evaluado la utilidad de estas técnicas para la detección del microoorganismo en los tejidos de peces enfermos.

En el año 2003, Cepeda y colaboradores describen un método molecular, rápido (menos de 4 h) y no tóxico, basado en la técnica de la PCR anidada para el diagnóstico de la tenacibaculosis causada por *T. maritimum*. Este método fue validado de acuerdo con los protocolos de validación ya descritos para patógenos de peces (Hiney, 1997; Hiney y Smith, 1998), mediante la realización de ensayos de especificidad y sensibilidad en el laboratorio, la identificación de las cepas utilizando en paralelo sistemas convencionales y otros protocolos de PCR y la realización de ensayos de campo utilizando DNA obtenido de tejidos de peces enfermos o de cultivos puros de cepas de *T. maritimum* aisladas durante el período 1992-2003. Posteriormente, Avendaño-Herrera y col (2004 b) han descrito un método no destructivo para la detección de *T. maritimum* en el mucus de peces. Sin embargo, dado que *T. maritimum* es un microorganismo característico de ambientes marinos y tiene capacidad de resistir la acción bactericida del mucus de dorada y rodaballo, la simple presencia del microorganismo en el mucus no necesariamente indicaría que los peces padecen la enfermedad.

En el caso de *T. maritimum* se han descrito también métodos basados en el análisis del genoma de utilidad en estudios epidemiológicos. Así, se ha demostrado que la técnica de amplificación aleatoria del DNA (RAPD) (Avendaño y col., 2004 c) y el

ribotipado (Pazos, 1997) permiten la diferenciación de las cepas en función de su origen y/o serotipo.

No se han descrito métodos serológicos o moleculares para el diagnóstico de la flexibacteriosis causada por *T. ovolyticum*. Si bien existen una serie de caracteres taxonómicos convencionales que permiten establecer diferencias entre *T. maritimum* y *T. ovolyticum* (Tabla 2). Tampoco se han establecido el tipo de pruebas que permitan diferenciar *T. maritimum* y *T. ovolyticum* de otras bacterias filamentosas capaces de producir enfermedad en peces marinos.

**Tabla 2.-** Características generales de *Tenacibaculum maritimum* y *T. ovolyticum.*

| Carácter | ***Tenacibaculum maritimum*** | ***Tenacibaculum ovolyticum*** |
|---|---|---|
| Movilidad deslizante | + | + |
| Pigmento tipo flexirrubina | - | - |
| Adherencia al agar | + | - |
| Absorción del rojo congo | + | - |
| Gram | - | - |
| Metabolismo O/F | O | O |
| Reducción de nitratos | + | + |
| Producción de | | |
| Catalasa | + | + |
| Citocromo oxidasa | + | + |
| Producción de $H_2S$ | - | - |
| Degradación de | | |
| Caseína | + | - |
| Gelatina | + | + |
| Almidón | - | - |
| Tirosina | + | + |
| Carboximetilcelulosa | - | - |
| Crecimiento en AOB con | | |
| 0,5% NaCl | - | - |
| 1,0% NaCl | - | - |
| 1,5% NaCl | - | - |
| Agua de mar | + | + |
| Crecimiento a | | |
| 5 ºC | - | + |
| 15 ºC | + | + |
| 20 ºC | + | + |
| 25 ºC | + | + |
| 30 ºC | + | + |
| 35 ºC | - | - |
| Contenido de G+C | 29,0-32,5 | 30,3-32,0 |

+, reacción positiva; -, negativa; ND, no determinado; O, oxidativo; AOB, caldo Anacker y Ordal (1955).

## 1.3.- Familia *Flavobacteriaceae*: el género *Tenacibaculum*

### 1.3.1- Taxonomía de las especies de *Tenacibaculum* patógenas de peces

La taxonomía del grupo de bacterias filamentosas no ha sido aún claramente establecida, produciéndose a lo largo del tiempo continuos cambios en función del conocimiento de nuevas características fenotípicas y moleculares de los microorganismos, creándose en ocasiones nuevos géneros y especies. Muestra de ello, son los diferentes nombres específicos utilizados a largo de estos años para designar a los agentes causales de la tenacibaculosis marina. Así, la similitud en las características fisiológicas, bioquímicas y genéticas de los aislados bacterianos obtenidos en Japón en 1977 con el género *Flexibacter*, permitió proponer el nombre de *Flexibacter marinus*, como una nueva especie del genero (Hikida y col., 1979). Sin embargo, la coincidencia del epíteto "*marinus"*, con el de *Vibrio marinus*, especie ya vigente en ese momento, lleva a los autores a proponer el nombre de *Flexibacter maritimus* que no sería aceptado hasta 1986 (Wakabayashi y col., 1986), quedando como cepa tipo la NCIMB 2154. Reinchenbach (1989) intenta una nueva combinación para *Flexibacter marinus*, y propone el nombre de *Cytophaga marina* como una nueva especie, incluyendo la cepa NCIMB 2153 como cepa tipo. Estudios de hibridación DNA-DNA demuestran posteriormente que ambas cepas corresponden a la misma especie (Bernardet y Grimont, 1989) y el nombre específico de *Flexibacter maritimus* prevalece frente a anteriores propuestas (Holmes, 1992).

Estudios recientes, basados en el análisis de las secuencias nucleotídicas del gen de la DNA girasa (*gyr*B), han desplazado al microorganismo junto con la especie *Flexibacter ovolyticus*, a un nuevo género "*Tenacibaculum*", y se establecen como nuevas especies "*Tenacibaculum maritimum"* manteniendo la cepa NCIMB 2154 como cepa tipo de la especie (Suzuki y col., 2001) y *T. ovolyticum* con la cepa NCIMB 13127 como cepa tipo de la especie. Al mismo tiempo se describen dos nuevos taxones dentro del género *Tenacibaculum*, la especie *T. mesophilum* que tiene como cepa tipo un microorganismo aislado de un homogeneizado de esponjas y la especie *T. amylolyticum* que tiene como cepa tipo una bacteria aislada de macroalgas en Japón. Posteriormente, Frette y col., (2004) describe *T. skagerrakense* de origen pelágico y aislada en la Bahía de Skagerrak (Dinamarca). Yoon y col., (2005) introducen la especie *T. lutimaris* aislada

de sedimentos de estuarios en Corea del Sur. Asimismo, *T. litoreum* y *T. aestuarii* se aislan por primera vez en el año 2006 a partir de sedimentos de estuarios en Corea del Sur (Choi y col., 2006; Jung y col., 2006). Más recientemente, se ha descrito la especie *T. litopenaei*, que incluye a microorganismos aislados de tanques de cultivo de gamba en Taiwán (Sheu y col., 2007) aunque no se aporta información sobre su posible interés como patógeno en acuicultura (Tabla 3). La figura 3 muestra la relación filogenética entre las especies de *Tenacibaculum* reconocidas en base al análisis de las secuencias del ARNr 16S.

**Tabla 3.** Características diferenciales de las cepas tipo del género *Tenacibaculum* 1, *T. mesophilum* MBIC 1140$^{T}$; 2, *T. lutimaris* KCTC 12302$^{T}$; 3, *T. skagerrakense* ATCC BAA-458$^{T}$; 4, *T. amylolyticum* MBIC 4355$^{T}$; 5, *T. ovolyticum* IAM 14318$^{T}$; 6, *T. maritimum* NCIMB 2154$^{T}$, 7, *T. litoreum* JCM 13039$^{T}$; 8, *T. aestuarii* KCTC 12569$^{T}$; 9, *T. litopenaei* BCRC 17590$^{T}$. Datos de Wakabayashi y col.,.1986; Hansen y col., 1992; Suzuki y col., 2001; Bernardet y col., 2002; Frette y col., 2004; Yoon y col., 2005; Choi y col., 2006 ; Jung y col., 2006; Sheu y col., 2007. Abbreviaturas; +, resultado positivo; -, resultado negativo; v, crecimiento débil; NT, no determinado; NG, ausencia de crecimiento.

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
|---|---|---|---|---|---|---|---|---|---|
| Origen | Homogenado de esponjas | Sedimento de estuario | Pelágica | Macroalgas | Huevos de Halibut | Peces enfermos | Sedimento de estuario | Sedimento de estuario | Tanque de cultivo de gambas |
| Tamaño de la célula (µm) | 1.5-10x0.5 | 2-10x0.5 | 2-15x0.5 | 2-5x0.4 | 2-20x0.5 | 2-30x0.5 | 2-35x0.3-0.5 | 2.0-3.5x0.3 | 2-10x0.3-0.5 |
| Movilidad | + | + | - | + | + | + | + | + | + |
| Crecimiento: | | | | | | | | | |
| %NaCl | 1-7 | <8 (2-3) | NG | 3 | NG | NG | 3-5 | <7 | 2-10 |
| Agua de mar (%) | 10-100 | 25-175 | 25-150 | 50-100 | 70-100 | 30-100 | 25-250 | NT | NT |
| Rango de temperatura (ºC) | 15-40 | 10-39 | 10-40 | 20-35 | 4-25 | 15-34 | 5-40 | 9-41 | 10-39 |
| Temperatura óptima (ºC) | 28-35 | 30-37 | 25-37 | 27-30 | NT | 25-30 | 35-40 | 30–37 | 28-37 |
| Rango pH | 5.3-9 | 7.0-8.0 | 6.0-9.0 | 5.3-8.3 | 5.9-8.6 | 5.9-8.6 | 6.0-10.0 | 5.5-8.5 | 5-10 |
| Reducción de nitratos | - | - | + | V | + | + | + | - | - |

**Tabla 3**. *(continuación)*.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Degradación de | | | | | | | | | |
| Almidón | - | - | + | + | - | - | + | - | + |
| Gelatina | + | + | NT | + | + | + | + | + | + |
| Quitina | + | NT | - | - | + | - | NT | NT | - |
| Tween 80 | + | - | - | + | + | + | + | + | - |
| Utilización de fuentes de carbono | | | | | | | - | | |
| D (+)-Sacarosa | - | - | + | - | - | - | - | - | - |
| D (-)-Ribosa | - | NT | NT | - | - | - | - | - | NT |
| D (+)-Galactosa | NT | NT | NT | NT | NT | - | - | - | - |
| D (+) –Glucosa | NT | - | + | NT | NT | - | - | - | + |
| L-prolina | + | - | + | + | - | - | + | - | + |
| L-glutamato | + | - | + | + | - | V | - | - | + |
| L-tirosina | NT | NT | NT | NT | NT | - | NT | NT | NT |
| DNA G+C (mol%) | 31.6-32.0 | 32.3-32.8 | 35.2 | 31.6-32.0 | 30.3-32.0 | 31.3-32.5 | 30 | 33.6 | 35.2 |

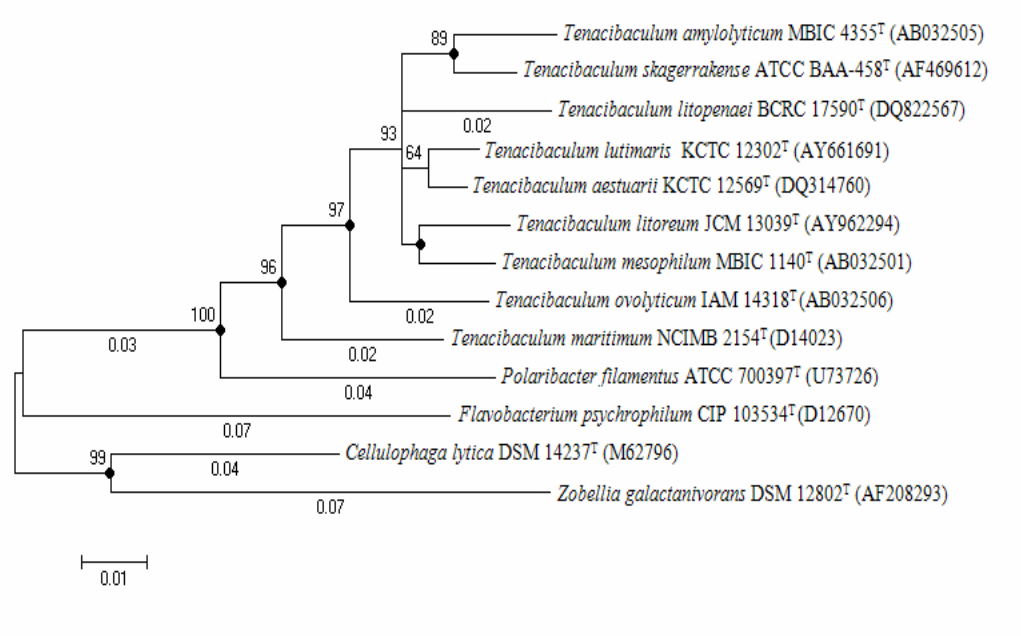



**Fig. 3.-** Árbol filogenético mostrando la relación entre las distintas especies del género *Tenacibaculum*. El análisis se basa en el ARNr 16S usando el método de neighbor-joining (Saitou y Nei, 1987) con el parámetro de kimura-2 (Kimura, 1980). Los valores de la replicación de los cálculos se muestran en porcentajes en los nodos. (Sólo se muestran porcentajes mayores al 50% para 1000 replicaciones). Los círculos en los nodos indican que éstos coinciden con los obtenidos calculando el árbol mediante el método de máxima parsimonia (Fitch, 1971).

### 1.3.2- Caracteres básicos para la descripción de nuevos taxones de la familia *Flavobacteriaceae*

La familia *Flavobacteriaceae* fue propuesta por Jooste (1985) e incluida en la primera edición del Manual Bergey de Bacteriología Sistemática pero el taxón no se describió formalmente (Holmes, 1997). Posteriormente el nombre de la familia fue validado (Reinchenbach, 1989) y se publicó una descripción enmendada (Bernardet y col., 1996). Actualmente la familia *Flavobacteriaceae* está compuesta por más de 40 géneros reconocidos y un elevado número de especies, algunas de las cuales incluyen microorganismos patógenos que causan importantes pérdidas económicas en acuicultura.

En el año 2002, Bernardet y colaboradores describieron una serie de criterios generales y de caracteres básicos para la descripción de nuevos taxones dentro de la familia *Flavobacteriaceae*. En primer lugar, es esencial definir la posición filogenética de la nueva especie propuesta en base a la comparación de la secuencia del gen ARNr 16S con secuencias de las cepas tipo de las especies pertenecientes al género al cual pertenece y a otras pertenecientes a la familia. Aunque Yamamoto y Harayama (1996) indicaron que el análisis filogenético basado en el estudio del gen *gyr*B puede tener un mayor grado de resolución que el basado en el ARNr 16S, Suzuki y col. (2001) demostraron que los dos métodos generan estructuras filogenéticas equivalentes en el complejo *Cytophaga-Flavobacterium-Bacteroides*.

Los árboles filogenéticos han de construirse empleando métodos suficientemente testados y reconocidos como el método del vecino más próximo, máxima parsimonia, UPGMA (Sneath y Sokal, 1973; Nei, 1987; Saitou y Nei, 1987). Además, la robustez de los árboles ha de evaluarse estadísticamente en base a criterios de bondad como el análisis de muestreo repetitivo (“bootstrap analysis”) (Felsntein, 1985).

Es obligatorio el depósito de la nueva secuencia del gen ARNr 16S en una base de datos reconocida, la inclusión del número de acceso de la secuencia en la descripción de la especie, y la designación de una cepa tipo y depósito de la misma en, al menos, dos colecciones de cultivo reconocidas.

Las características mínimas que han de ser evaluadas para la definición de nuevos géneros dentro de la Familia *Flavobacteriaceae* incluye: tipo de pigmento producido,

movilidad deslizante, requerimiento de sales para el crecimiento, tipo de metabolismo, determinación de la composición de ácidos grasos y del contenido en G+C (Bernardet y col., 2002). Las características mínimas que han de ser evaluadas para la descripción de nuevas especies dentro de los géneros incluidos en la Familia *Flavobacteriaceae* son: características fenotípicas, análisis genómico (hibridación DNA-DNA), análisis de las proteínas de la envoltura celular y de los ácidos grasos y estudio de la relación con los hospedadores (Bernardet y col., 2002).

## 1.4.- *Quorum sensing*: comunicación e interacción celular

### 1.4.1.- Moléculas señal o autoinductoras en bacterias

Históricamente, las bacterias habían sido consideradas como unidades independientes con una escasa capacidad para desarrollar funciones colectivas. Sin embargo, investigaciones realizadas desde la década de los 60 demostraron que las bacterias disponen de complejos mecanismos de comunicación intercelular capaces de generar y recibir mensajes de naturaleza química (Bassler y Losick, 2006; Williams y col., 2007). Este fenómeno es conocido como *quorum sensing* (QS) y se basa en el uso de molécula/s señal o autoinductoras (Fuqua y col., 1994). A través del uso de autoinductores, las bacterias pueden regular su comportamiento en función de la densidad de la población (Kievit e Iglesky, 2000; Williams y col., 2000, 2007). Cuando la densidad celular en el medio es suficiente, la concentración del autoinductor alcanza un valor umbral que permite a la bacteria detectar la existencia de masa crítica y, en respuesta a ello, activar o reprimir la expresión de genes diana (Fuqua y col., 1994; Williams y col., 2007). Para que una molécula pueda ser considerada como autoinductora deben cumplirse una serie de requisitos: i) la producción de la molécula debe ocurrir durante estadíos específicos de crecimiento o en respuesta a un cambio ambiental, ii) la molécula debe acumularse en el ambiente extracelular y debe poder ser reconocida por un receptor bacteriano, iii) cuando la concentración del autoinductor alcance el valor umbral debe estimularse una respuesta concertada, iv) la respuesta celular debe manifestarse de forma diferente a los cambios fisiológicos necesarios para metabolizar o detoxificar al autoinductor (Williams y col., 2007).

La señal de *quorum* no es algo homogéneo dentro del dominio *Bacteria*. Las bacterias Gram-negativas producen generalmente *N*-acil-L-homoserín lactonas (AHLs) que poseen una elevada diversidad conformacional, difiriendo en la longitud de cadena lateral. Las AHLs se identificaron por primera vez en bacterias bioluminiscentes marinas, donde tienen un papel fundamental en el control de la emisión de luz en simbiontes, como *Vibrio fischeri,* que constituye el paradigma de los circuitos de control genético por QS en Gram-negativas. El sistema de QS de *V. fischeri* consta de dos unidades: LuxI, la proteína encargada de la síntesis de la señal y LuxR, el regulador de respuesta encargado de detectarla. Se han descrito sistemas análogos a LuxI/LuxR en más de 50 especies distintas, incluyendo un gran número de patógenos humanos y de animales y plantas en los que se regulan procesos como la síntesis de antibióticos, transferencia de plásmidos, síntesis de polisacáridos, exoenzimas relacionados con la virulencia, maduración de biopelículas etc. Aproximadamente un 60% de las bacterias aisladas del suelo producen sustancias que presentan actividad en los bioensayos de detección de AHLs. En el medio marino, hasta el 10% de los aislados marinos producen AHLs, aunque este número es probablemente una subestima del número real (Rice y col., 1999).

En bacterias Gram-positivas la comunicación intercelular se realiza mediante la acción de pequeñas moléculas señal de naturaleza peptídica. Las moléculas señal se exportan activamente al medio y en algunos casos se modifican y truncan para interaccionar con los dominios externos de proteínas sensoras de membrana. La transducción de la señal se produce mediante una cascada de fosforilación que culmina en la activación de una proteína que modula la transcripción de los genes diana, controlando procesos como la secreción de factores de virulencia, esporulación y competencia.

Se han descrito otros sistemas de *quorum* denominados AI-2, en el que autoinductor es un diéster furanosil borato y AI-3/epinefrina-norepinefrina, cuya molécula señal es un compuesto aromático. El sistema AI-2 se identificó por primera vez en otro vibrio marino bioluminiscente, *V. harveyi.* Mientras que las AHLs y los autoinductores peptídicos son altamente específicos y se utilizan para la comunicación intercelular dentro de la misma especie, AI-2 se encuentran tanto en bacterias Gram-positivas como Gram-negativas, lo que sugiere que podría actuar como lenguaje químico interespecífico (Miller y Bassler, 2001; Federle y Bassler, 2003). El sistema AI-3 se detectó en *Escherichia coli* enteropatógenos de los serotipos O26:H11 y O111ac:H9, *Shigella* sp y *Salmonella* sp pero también en

microorganismos comensales como *E. coli*, *Klebsiella pneumoniae* y *Enterobacter cloacae*, lo cual sugiere que el sistema AI-3 puede desempeñar un papel en la comunicación interespecífica entre la microbiota intestinal. El sistema AI-3 puede tener un papel en la comunicación interreinos debido a que los sensores bacterianos pueden detectar, además de la molécula autoinductora, a las hormonas epinefrina y norepinefrina presentes en el tracto gastrointestinal y de este modo conocer el estado metabólico del hospedador (Reading y Sperandio, 2006).

### 1.4.2.- *Quorum sensing* y enfermedades infecciosas

Uno de los pasos claves en la mayoría de las infecciones bacterianas es la necesidad del patógeno de alcanzar una densidad suficiente para vencer los mecanismos de defensa del hospedador y establecerse. El control de la expresión de los factores de virulencia, de forma concertada con la densidad de la población, confiere al patógeno una ventaja de supervivencia significativa dado que el hospedador puede ser invadido antes de que pueda iniciar una respuesta de defensa.

Es conocido que diferentes bacterias patógenas regulan diferentes procesos fisiológicos, incluyendo la producción de factores de virulencia a través de sistemas de *quorum*. La capacidad de una población bacteriana para coordinar su estrategia de ataque al hospedador puede ser un componente esencial para el desarrollo de la infección, particularmente por patógenos oportunistas. Además, ciertas señales de *quorum* pueden actuar como determinantes de virulencia *per se*, gracias a su capacidad para modular la respuesta inmune del hospedador (Telford y col., 1998).

Los sistemas de regulación de la virulencia denso-dependientes no son demasiado conocidos en otras especies aunque sí existen evidencias para algunas bacterias Gram-negativas y Gram-positivas patógenas para el ser humano, como *Pseudomonas aeruginosa*, *Burkholderia cepacia* y *Staphyloccocus aureus* (Van Delden e Iglewsky, 1998; Parsek & Greenberg, 2000; Donabedian, 2003). Así, se ha demostrado que *P. aeruginosa* usa el sistema de quórum para activar varios genes responsables de la colonización y persistencia en el hospedador (Parsek & Greenberg, 2000; Donabediam, 2003). El sistema de *quorum* en *B. cepacia* regula positivamente la producción de proteasas y negativamente la síntesis de sideróforos. Además, dado que *B. cepacia* puede utilizar AHLs exógenas producidas por

otras bacterias, se ha sugerido que la comunicación interespecies a través de señales de *quorum* puede también jugar un papel en la regulación de la patogenicidad. Con respecto a las bacterias Gram-positivas, es conocido que la patogenicidad de *S. aureus* depende de la expresión de factores celulares (colágeno, proteína A…) y extracelulares (lipasas, proteasas, hemolisinas…) cuya síntesis es regulada por señales de *quorum* (Donabediam, 2003). Los circuitos de QS mediados por AHLs también se han descrito en bacterias patógenas de peces pertenecientes a las especies *Vibrio harveyi, Photobacterium fischeri (Vibrio fischeri), Listonella anguillarum, Aeromonas hydrophila*, *A. salmonicida*, *V. parahaemolyticus*, *V. vulnificus* y *Yersinia ruckeri* (Buch y col., 2003; Buchholtz y col., 2006; Bruhn y col., 2005; Manefield y col., 2000) y existen evidencias de que la expresión de algunos factores de virulencia (proteasas, sideroforos, toxinas) y formación de biofilm están reguladas por AHLs en algunas de estas especies (Swift y col., 1997; 1999; Lilley y Bassler, 2000; Manefield y col., 2000; McDougald y col., 2000, Temperano y col., 2001). Además, en los tejidos de peces infectados experimentalmente con *L. anguillarum* (Buch y col., 2003; Buchholtz y col., 2006) y *Y. ruckeri* (Kastbjerg y col., 2006) se ha detectado la presencia de acil-homoserín lactonas, lo cual sugiere que las señales de *quorum* pueden jugar un papel en el proceso infeccioso. Recientemente, Romero y col., (2008) han descrito la presencia de AHLs (C4-HSL, C8-HSL, C12-HSL y C14-AHLs) en los sobrenadantes de los cultivos de distintas cepas de *Tenacibaculum maritimum* y su correlación con la formación de biofilm por esta bacteria. Este estudio representa la primera descripción de producción de AHLs *in vitro* en microorganismos no pertenecientes a las Proteobacteria.

### 1.4.3.- Inhibición de *quorum sensing*

Debido a que muchas bacterias utilizan señales de *quorum* para la regulación de numerosas funciones fisiológicas, incluyendo las relacionadas con la patogenicidad, existe un enorme interés en el diseño e implementación de estrategias de inactivación de los sistemas de QS, también denominadas *quorum quenching* (QQ) (Zhang, 2003; Otero Casal y col., 2005), que puedan funcionar como nuevas fórmulas de lucha antimicrobiana. Los compuestos inhibidores de *quorum sensing* pueden constituir un nueva generación de agentes antimicrobianos con aplicación en diferentes áreas, incluyendo la medicina

(humana y veterinaria), agricultura y acuicultura (Hentzer y Givskov, 2003; Zhang, 2003; Uroz y col., 2003; Defoirdt y col., 2004; Rash y col., 2004; Sio y col., 2006; Bai y col., 2008). Mientras que los agentes antimicrobianos tradicionales resultan en la muerte celular del patógeno, la utilización de sistemas que alteren los sensores de QS adopta una estrategia menos agresiva, atenuando la virulencia del patógeno de modo que no sea capaz de adaptarse al ambiente del hospedador y pueda ser eliminado por las defensas innatas de éste (Otero Casal y col., 2005).

Existen dos tipos de estrategias de inactivación del QS: la primera está encaminada a evitar la generación de la señal, mientras que el segundo tipo interceptaría la señal para evitar que fuese detectada (Figura 4) (Whitehead y col., 2001; Hentzer y Givskov, 2003; Defoirdt y col., 2004; Otero Casal y col., 2005).

La mayoría de las bacterias Gram-negativas que producen AHLs como moléculas señal de QS utilizan proteínas homólogas a la LuxI producida por *P. fisheri* (*V. fisheri*) para la síntesis de AHLs. Esta reacción implica el uso de S-adenosil-metionina (SAM) como sustrato para generar el anillo de la lactona y una proteína transportadora (ACP, Acyl-Carrier-Protein) unida al ácido graso como precursor de la cadena acilada. El bloqueo de la síntesis de la señal se puede producir mediante (Fig 4): i) la inhibición de la unión del ácido graso con la ACP para formar el complejo acil-ACP o por ii) la inhibición de las sintetasa de AHL o de los posibles transportadores de membrana de la señal, en el caso de que existan. Se ha demostrado que algunos análogos de la SAM (S-adenosilhomocisteína, S-adenoilcisteína) y ciertos macrólidos son potentes inhibidores de la síntesis de AHLs (Hentzer & Givskov, 2003; Defoirdt y col., 2004; Otero Casal y col., 2005).

En cuanto a la estrategia de interceptación de la señal puede ocurrir mediante su degradación química, su secuestro por proteínas específicas o la utilización de homólogos que bloqueen a la proteína receptora. (Fig 4).

Numerosas bacterias del suelo, principalmente del género *Bacillus*, tienen capacidad para bloquear de forma específica el sistema de *quorum* de bacterias Gram-negativas, mediante la acción de enzimas denominados lactonasas que actúan hidrolizando el anillo lactona. Esta estrategia puede conferir a la bacteria una ventaja selectiva sobre otros microorganismo que compiten por su mismo hábitat (Dong y col., 2002). Algunas bacterias como *Agrobacterium tumefaciens* usan las lactonasas como método de control de sus mecanismos de virulencia dependientes

de *quorum* (Zhang y col., 2002; Zhang, 2003; Sio et al., 2006). Un segundo tipo de enzimas inactivadoras de *quorum* ha sido identificado en otra bacteria Gram-negativa aislada del suelo, *Variovorax paradoxus* (antes *Alcaligenes paradoxus*), que utiliza las AHLs como única fuente de energía y nitrógeno (Leadbetter y Greenberg, 2000). En este caso se trata de una AHL-acilasa, que libera el anillo lactona como producto de degradación, utilizándose el ácido graso como fuente de energía. Una enzima similar, denominado AiiD, que libera la cadena lateral del anillo lactona, ha sido clonada de otra β-proteobacteria formadora de biopelículas, *Ralstonia* sp. (Lin y col., 2003; Taga y Bassler, 2003, Otero Casal y col., 2005). Huang y col., (2003) han demostrado la producción de acilasas con especificidad para las AHLs de cadena larga en *Pseudomonas* sp. aisladas del suelo.

En organismos superiores también se ha demostrado la producción de compuestos con capacidad para inactivar el QS. El ejemplo más conocido es el del alga roja *Delisea pulchra,* endémica de Australia. Esta alga produce furanonas halogenadas, compuestos con estructura similar a las de las AHLs, que interceptan la señal de *quorum* bloqueando los receptores de AHL. Las furanonas funcionarían como antagonistas de la señal de *quorum* desplazando las AHLs de su receptor de la familia LuxR. Resultados más recientes indican que es posible que la interacción de las furanonas con el sensor tipo LuxR no consista en el desplazamiento del sitio de unión, sino que afectaría a la vida media de la proteína, ya que no forma complejos estables con ella (Zhang, 2003). Recientemente se han descrito dos moléculas aisladas del briozoo marino *Flustra foliacea* que inhiben la comunicación intercelular mediante AHLs, aunque se desconoce el mecanismo de acción y su estructura no recuerda las AHLs (Peters y col., 2003). Las haloperoxidasas del alga *Laminaria digitata* pueden inactivar las AHLs gracias a la formación de OHBr, que al igual que otros biocidas halógenos (OHCl) inactivan las 3-oxo-AHL (Borchardt y col., 2001). Algunos exudados de plantas terrestres, como el guisante, también producen inhibición del QS pero se desconoce la naturaleza y mecanismo de acción del inhibidor (Zhang, 2003). Otras plantas superiores, como el tomate, producen

compuestos no identificados que interactúan con los sistemas de QS dependientes de AHLs (Whitehead y col., 2001 b).

A pesar del innegable atractivo que presenta la utilización de estrategias de inhibición del *quorum* como método de lucha frente a las enfermedades infecciosas se han descrito limitaciones para su aplicación como terapia anti-infectiva. Así, se ha descrito (Zhu y col., 1998) que algunas bacterias pueden solventar el bloqueo de las señales de *quorum* mediante la sobre-expresión de los genes de QS. Además, la mayoría de los sistemas de disrupción de *quorum* descritos hasta el momento bloquean los sistemas de *quorum* de microorganismos patógenos y no patógenos, pudiendo afectar adversamente procesos favorables regulados por *quorum*. Esta falta de especificidad de los sistemas de QQ podría limitar su uso sólo al control de patógenos.

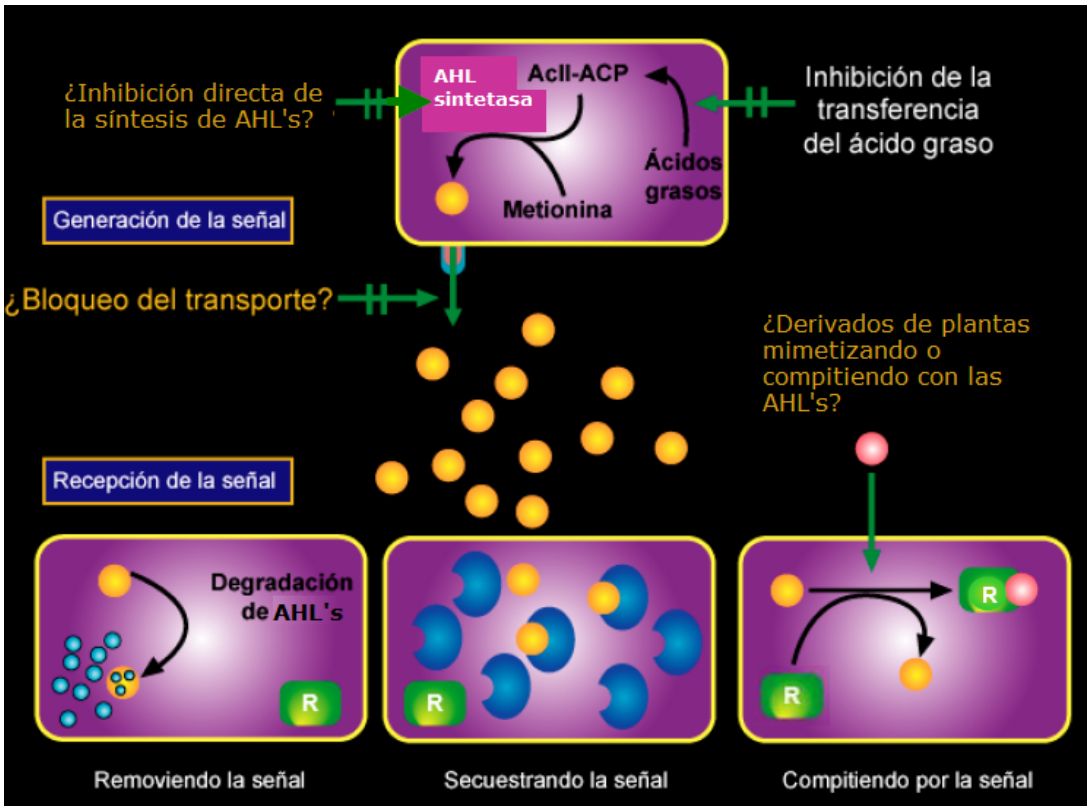



**Fig. 4**.- Posibles estrategias de inactivación de la señal de quórum dependiente de AHLs (Modificado de Loh y col., Curr. Opin. Plant Biol., 2002). Las estrategias para las que no se ha establecido el mecanismo de acción se indican entre signos de interrogación.

# Capítulo II. Objetivos

# Capítulo II. OBJETIVOS

La tenacibaculosis o flexibacteriosis marina es una enfermedad de etiología bacteriana causante de importantes pérdidas económicas en los cultivos de peces marinos a nivel mundial. *Tenacibaculum maritimum* es considerado el principal agente causal de mortalidades en la mayor parte de las especies en cultivo y en peces salvajes, mientras que *T. ovolyticum* se considera como patógeno oportunista de huevos y larvas de halibut atlántico. Nuestro grupo ha aislado a partir de rodaballos y lenguados enfermos y del agua de mar de los tanques de cultivo otras bacterias filamentosas patógenas para peces y que muestran caracteres fenotípicos similares a *T. maritimum*. Con el fin de esclarecer la posición taxonómica de estos microorganismos y establecer criterios útiles para el diagnóstico y la prevención de la enfermedad, en el presente trabajo nos hemos propuesto los siguientes objetivos:

1. Determinación de la posición taxonómica y estudio de la virulencia para peces de bacterias filamentosas aisladas de sistemas de cultivo de rodaballo y lenguado.

2. Establecimiento de criterios útiles para la diferenciación de especies del género *Tenacibaculum* causantes de tenacibaculosis marina.

3. Analizar las relaciones serológicas entre los aislados con el fin de determinar la posible existencia de serotipos predominantes asociados a las mortalidades.

4. Estudio del potencial inmunogénico de antígenos celulares y extracelulares de *Tenacibaculum* sp. patógenas para peces.

5. Detección y caracterización de señales de “quorum sensing” en *Tenacibaculum* sp y estudio de su papel en la patogénesis de la tenacibaculosis.

Para llevar a cabo estos objetivos se utilizaron las siguientes cepas de *Tenacibaculum maritimum*, *T. ovolyticum*, *T. litoreum* y las bacterias aisladas de epizootias en piscifactorías de nuestra área.

Tabla 4.- Cepas de *Tenacibaculum* utilizadas en el presente estudio

| Cepa | Especie | Origen | Año de aislamiento |
|---|---|---|---|
| NCIMB2153 | *Tenacibaculum maritimum* | "Blackhead seabream" (*Acanthopagrus schlegeli,* Blecker), Japón | 1976 |
| Lyl-1 | *T. maritimum* | "Blackhead seabream" (*A. schlegeli,* Blecker), Japón | 1985 |
| NCIMB 2154 | *T. maritimum* | "Japanese seabream" (*Pagrus major,* Temminck y Schlegel), Japón | 1977 |
| FPC394 | *T. maritimum* | "Japanese seabream" (*P. major,* Temminck y Schlegel), Japan | |
| NCIMB 2158 | *T. maritimum* | Lenguado (*Solea solea,* L.), Reino Unido | 1981 |
| JIP21/91 (2) | *T. maritimum* | Lubina (*Dicentrarchus labrax*, L.), Francia | 1991 |
| JIP32/91 | *T. maritimum* | Lubina (*D. labrax*, L.), Francia | 1991 |
| GBF 8601 | *T. maritimum* | "Bastard halibut" (*Paralychthys olivaceous*, Temminck y Schlegel), Japón | 1986 |
| DBA 4 a | *T. maritimum* | "Japanese amberjack" (*Seriola quinqueradiata,* Temminck y Schlegel), Japón | 1986 |
| FES342/01 | *T. maritimum* | Dorada (*Sparus aurata,* L.) | 2001 |
| DOB 102 | *T. maritimum* | Dorada (*S. aurata,* L.) | 2002 |
| UCD6F | *T. maritimum* | Anchoveta californiana Engraulis mordaz, Girard), EEUU | 1994 |
| UCD WSB-1b | *T. maritimum* | "White sea bass" (*Atractoscion mobilis*, Ayres), EEUU | |

| | | | |
|---|---|---|---|
| LL01 7.3.1 | *T. maritimum* | Lenguado (*Solea senegalensis,*Kaup), España | 2001 |
| LL01 8.3.8 | *T. maritimum* | Lenguado (*S. senegalensis,*Kaup), España | 2001 |
| LL01 10.5.3 | *T. maritimum* | Lenguado (*S. senegalensis,*Kaup), España | 2002 |
| LL03 6.2.1 | *T. maritimum* | Lenguado (*S. senegalensis,*Kaup), España | 2003 |
| LL03 7.5.2 | *T. maritimum* | Lenguado (*S. senegalensis,*Kaup), España | 2003 |
| LC0710.3.1 | *T. maritimum* | Lenguado (*S. senegalensis,*Kaup), España | 2007 |
| LC0710.1.2 | *T. maritimum* | Lenguado (*S. solea,*L.), España | 2007 |
| JIP 10/97 | *T. maritimum* | Rodaballo (*Psetta maxima,* L.), Francia | 1997 |
| JIP 31/99 | *T. maritimum* | Rodaballo (*P. maxima,* L.), Francia | 1999 |
| RA107.1 | *T. maritimum* | Rodaballo (*P. maxima,* L.), Francia | 2007 |
| Tm Chile | *T. maritimum* | Rodaballo (*P. maxima,* L.), Chile | 1998 |
| C02 4.1.3 | *T. maritimum* | Rodaballo (*P. maxima* L.) España | 2002 |
| C03 6.1.3 | *T. maritimum* | Rodaballo (*P. maxima* L.) España | 2003 |
| C04 3.2.1 | *T. maritimum* | Rodaballo (*P. maxima* L.) España | 2004 |
| LPV1.7 | *T. maritimum* | Rodaballo (*P. maxima* L.) España | 1995 |
| C02 8.1.1 | *T. maritimum* | Rodaballo (*P. maxima* L.) España | 2002 |
| C02 11.2.1 | *T. maritimum* | Rodaballo (*P. maxima* L.) España | 2002 |
| C03 11.2.1 | *T. maritimum* | Rodaballo (*P. maxima* L.) España | 2003 |
| LS95 2.2.1 | *T. maritimum* | Sole (*S. solea* L.) España | 1995 |
| LL04 11.1.1 | *Tenacibaculum* sp. | Lenguado (*S. senegalensis*, Kaup) España | 2004 |
| LC06 11.5.1 | *Tenacibaculum* sp. | Lenguado (*S. senegalensis*, Kaup) España | 2006 |
| LC07 3.2.2 | *Tenacibaculum* sp. | Lenguado (*S. senegalensis*, Kaup) España | 2007 |
| ITd07.2 | *Tenacibaculum* sp. | Rodaballo (*P. maxima* L.) España | 2007 |

| | | | |
|---|---|---|---|
| ITd07.4 | *Tenacibaculum* sp. | Rodaballo (*P. maxima* L.) España | 2007 |
| ITd07.5 | *Tenacibaculum* sp. | Rodaballo (*P. maxima* L.) España | 2007 |
| A22.2 | *Tenacibaculum* sp. | Agua de tanques de cultivo, España | 2003 |
| R006.0.1 | *Tenacibaculum* sp. | Rodaballo (*P. maxima* L.) España | 2000 |
| R006.0.3 | *Tenacibaculum* sp. | Rodaballo (*P. maxima* L.) España | 2000 |
| CLT07.1 | *Tenacibaculum* sp. | Rodaballo (*P. maxima* L.) España | 2007 |
| LC07 10.2.2 | *Tenacibaculum* sp. | Lenguado (*S. senegalensis*, Kaup) España | 2007 |
| A37.1 | *Tenacibaculum* sp. | Agua de tanques de cultivo, España | 2003 |
| A13.3 | *Tenacibaculum* sp. | Agua de tanques de cultivo, España | 2003 |
| A35.1 | *Tenacibaculum* sp. | Agua de tanques de cultivo, España | 2003 |
| LL04 12.1.7 | *Tenacibaculum* sp. | Lenguado (*S. senegalensis*, Kaup) España | 2004 |
| 567/06.1 | *Tenacibaculum* sp. | Lubina (*D. labrax*, L.) España | 2006 |
| 567/06.2 | *Tenacibaculum* sp. | Lubina (*D. labrax*, L.) España | 2006 |
| 567/06.3 | *Tenacibaculum* sp. | Lubina (*D. labrax*, L.) España | 2006 |
| DSM 18103 | *Tenacibaculum ovolyticum* | Huevos de halibut (*Hippoglossus hippoglossus*, L.) Noruega | 1989 |
| JCM 13039 | *Tenacibaculum litoreum* | Sedimentos de estuario, Corea | 2006 |

# Capítulo III. Aislamiento de nuevas bacterias filamentosas patógenas para peces marinos.

# III A. Publicaciones

**Artículo nº1**. Piñeiro-Vidal, M., Centeno-Sestelo, G., Riaza, A. & Santos, Y. (2007) Isolation of pathogenic *Tenacibaculum maritimum*-related organisms from diseased turbot and sole cultured in the Northwest of Spain. *Bulletin of European Association of Fish Pathologists* **27**, 29-35.

Bull. Eur. Ass. Fish Pathol., 27(1) 2007, 29

# Isolation of pathogenic *Tenacibaculum maritimum*-related organisms from diseased turbot and sole cultured in the Northwest of Spain

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**Abstract**

The present study deals with the isolation and characterization of filamentous bacteria, phenotypically related to *Tenacibaculum maritimum,* from Senegalese sole, (*Solea senegalensis*, Kaup) and turbot (*Scophthalmus maximus,* L.) cultured in the Northwest of Spain. Diseased fish showed the typical signs observed in fish affected by marine flexibacteriosis (eroded mouth, rotten fins, shallow skin lesions and paleness of internal organs). The isolated bacteria showed similar morphological, physiological and biochemical characteristics to the *Tenacibaculum maritimum* strains used as reference in the study. However, results of PCR and serological analysis indicated that the isolated bacteria were antigenically and genetically different from *T. maritimum* species. Pathogenicity assays demonstrated that these *T. maritimum*-like bacteria are virulent for turbot and sole with bacterial doses ranging from 9.0 x$10^5$ to 9.0 x $10^7$ colony forming units (CFU) per fish.

## Introduction

Marine flexibacteriosis is considered a potential limiting factor for the culture of economically important marine fish species such as turbot (*Scophthalmus maximus*), Dover sole (*Solea solea*), Senegalese sole (*Solea senegalensis*), sea bass (*Dicentrarchus labrax*), Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) (McVicar & White, 1979 & 1982; Devesa et al., 1989; Bernardet et al, 1990; Pazos et al. 1993; Bernardet, 1997; Cepeda & Santos, 2002; Cepeda et al, 2003), causing serious mortalities in farms in many countries (Santos et al., 1999). External pathological signs of this disease are eroded mouths, ulcerated skin lesions, and fins and tail rot. Infected tissues can appear pale-yellow due to the presence of large number of bacteria. Some affected fish may have shallow skin lesions or darkening of tissue between caudal and marginal fin rays, loss of epithelial surface and haemorrhage in exposed dermal tissue (Bernardet et al., 1990; Alsina & Blanch, 1993; Pazos et al., 1993). The filamentous organism *Tenacibaculum maritimum* (Suzuki et al., 2001), is the causative agent of flexibacteriosis in marine fish.

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| Strains | Source | Antigen | Slide agglutination test with antisera against[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *T. maritimum* | | | | Strains present study | | | |
| | | | (O1) | (O2) | (O3) | (O4) | R00.6.0.1 | R01.6.8.1 | LL04.11.1.1 | A37.1 |
| ***T. Maritimum*** | | | | | | | | | | |
| NCIMB2153 O1) | *Acanthopagrus schlegeli*, Bleeker, Japan | FKC | + | + | + | + | - | - | - | - |
| | | "O" | + | - | - | - | - | - | - | - |
| LL018.3.8 (O1) | *Solea senegalensis* Kaup, Spain | FKC | + | + | + | + | - | - | - | - |
| | | "O" | + | - | - | - | - | - | - | - |
| LPV1.7 (O2) | *Scophthalmus macimus*, L. | FKC | + | + | + | + | - | - | - | - |
| | | "O" | - | + | - | - | - | - | - | - |
| LM02.7.5.4 (O3) | *S. maximus*, L., Spain | FKC | + | + | + | + | - | - | - | - |
| | | "O" | - | + | - | - | - | - | - | - |
| Q02.10.1.2 (O4) | *S. maximus*, L., Spain | FKC | + | + | + | + | - | - | - | - |
| | | "O" | - | - | - | + | - | - | - | - |
| **Isolates present study** | | | | | | | | | | |
| 2/8 strain [b] | *S. maximus*, L., Spain | FKC | - | - | - | - | + | - | - | - |
| | | "O" | - | - | - | - | + | - | - | - |
| 1/8 strain [c] | *S. maximus*, L., Spain | FKC | - | - | - | - | - | + | - | - |
| | | "O" | - | - | - | - | - | + | - | - |
| 1/4 strains [d] | *S. senegalensis*, Kaup Spain | FKC | - | - | - | - | - | - | + | - |
| | | "O" | - | - | - | - | - | - | + | - |
| 4/11 strains [e] | Sea water | FKC | - | - | - | - | - | - | - | + |
| | | "O" | - | - | - | - | - | - | - | + |
| 1/11 strains [c] | Sea water | FKC | - | - | - | - | - | + | - | - |
| | | "O" | - | - | - | - | - | + | - | - |

[a] Strong and rapid agglutination was registered as positive. [b] Strains typed with antiserum R00.6.0.1 were: R006.0.1 and R006.0.3; [c]Strains typed with antiserum R01.6.8.1 were: R01.6.8.1 and A52.1. [d] Strains typed with antiserum LL04.11.1.1 was: LL04.11.1.1, [e] Strains typed with antiserum A37.1 were: A13.3, A35.1, A37.1 and A38.1.

**Table 1.** Results of slide agglutination test with FKC and O antigen of *T. maritimum* and related bacteria and rabbit whole-cell antisera.

Bull. Eur. Ass. Fish Pathol., 27(1) 2007, 31

In Galicia (Northwest of Spain) flexibacteriosis is a significant disease problem for the culture of turbot and sole in landbased units. *Tenacibaculum maritimum* of serotypes O1, O2, and O3 and to a lower extent the serotype O4, have repeatedly been isolated from diseased fish (Pazos et al, 1993; Pazos, 1997; Santos et al, 1999; Cepeda & Santos, 2002; Cepeda et al, 2003). However, we have recently isolated other filamentous gliding bacteria associated with disease in juveniles and adult stages of turbot and sole as well as from seawater. These microorganisms were usually isolated in mixed cultures with *T. maritimum*, *Vibrio pelagius*, and *Vibrio splendidus*.

In the present study, the biochemical, serological and molecular characteristics as well as the virulence potential of a group of filamentous gliding bacteria isolated from marine fish and seawater were compared with those of *T. maritimum*.

## Materials and methods

### *Bacterial strains and phenotypic characterization*

In the present work we used twenty three strains isolated from diseased fish (12 strains) and seawater from fish holding tanks (11 strains) in the Norwest of Spain (Table 1). Reference strains of *Tenacibaculum maritimum* were obtained from the National Collection of Marine and Industrial Bacteria (NCIMB, Aberdeen, Scotland) and our own collection. The strains were routinely cultured on *Flexibacter maritimus* Medium (FMM) agar (Pazos et al., 1996) and incubated at 25°C for 24-48h. Stock cultures of the strains were stored at -70°C in Microbank™ tubes (Prolab Diagnostics, Ontario, Canada).

Bacterial isolates were identified using morphological, physiological and biochemical tests and API ZYM (Biomerieux) as previously described (Bernardet et al., 1990; Pazos et al., 1993; Pazos, 1997; Cepeda & Santos, 2002). Phenotypic profiles were compared to those of the strains of *T. maritimum* used as reference in the present study.

### *Serological characterisation*

Serological identification was performed using slide agglutination test as previously described (Santos et al., 1995). Sera were raised in rabbits following the procedures of Sorensen & Larsen (1986). Antigens consisted of inactivated whole cell of the type strain of *T. maritimum* NCIMB2153 and three Spanish isolates, LPV1.7, LMO2.7.5.4 and Q0210.1.2, representing the serotypes O1, O2, O3 and O4, respectively. The test was carried out using both formalin-killed cells (FKC) and "O" antigens and the rabbit whole cell antisera. Bacterial isolates that could not be typed by the *T. maritimum* antisera were retested with sera against representative strains of filamentous bacteria isolated from fish (R006.0.1, R01.6.8.1 and LL04.11.1.1) and seawater (A37.1). Strong and rapid agglutination was registered as a positive result and no or only a weak agglutination ocurring after 1 to 2 min as a negative reaction. Auto-agglutination (spontaneous agglutination) control was carried out by mixing equal volumes of antigen suspension and sterile saline solution instead of antiserum.

### *PCR-based identification of the isolates*

PCR amplifications were carried out using the species-specific primers MAR1 and MAR2 described by Bader & Shotts (1998) and the commercial kit Ready-to-go PCR beads

Bull. Eur. Ass. Fish Pathol., 27(1) 2007, 32

| Strains | Virulence for fish [a] | | |
|---|---|---|---|
| | Challenge dose (cfu fish $^{-1}$) | Turbot Mortality (%) | Sole Mortality (%) |
| *Tenacibaculum maritimym* | | | |
| LL01.8.3.8 (O1) | $2.0 \times 10^6$ | 90 | 100 |
| LPV1.7 (O2) | $3.0 \times 10^7$ | 60 | 90 |
| LM027.5.4 (O3) | $9.0 \times 10^5$ | ND | 100 |
| **Present study** | | | |
| LLO4.11.1.1 | $9.0 \times 10^7$ | 85 | 100 |
| R01.6.8.1 | $2.1 \times 10^7$ | 100 | 100 |
| R00.6.0.1 | $3.0 \times 10^7$ | 100 | 100 |
| A37.1 | 1.2 x 107 | 100 | ND |

[a] Virulence assays were performed by intraperitoneal inoculation of a single bacterial dose (between $10^6$ to $10^8$ cells per fish) in fish with an average weight of 10 g. ND, Not determined.

**Table 2.** Virulence for turbot and sole of *T. maritimum* and *T. maritimum*-related bacteria.

(Amersham Pharmacia Biotech, Piscataway, USA) as previously described (Cepeda et al., 2003). Briefly, one pmol of primers and 0.5µl of DNA template extracted from bacterial suspensions were added to PCR beads. The reaction mixtures were subjected to 40 amplification cycles in a Mastercycler personal thermal cycler (Eppendorf). The cycling conditions were 40 cycles of denaturation (94°C for 2 s), annealing (54°C for 2 s), and extension (72°C for 10 s). A preheating step at 94°C for 2 min and a final extension step consisting of 4 min at 72°C were carried out. The amplified products were analysed using vertical agarose electrophoresis and methylene blue staining method (VAGE/MeB) (Cepeda and Santos, 2000).

*Virulence test*

Representative strains of *T. maritimum* (LPV1.7, LMO2 7.5.4 and LL01.8.3.8) and *T. maritimum*-like bacteria (LL04.11.1.1, R00.6.0.1, R01.6.8.1, and A37.1) were tested for pathogenicity in turbot and sole with average weight of 10 g. Virulence assays were performed by intraperitoneal inoculation of a single bacterial dose (between $10^5$ to $10^8$ cells per fish) as previously described (Magariños et al., 1995). Mortalities were recorded daily for a 21 days period and were considered to be due to the inoculated strains if it was recovered from the internal organs of dead fish in pure culture.

## Results and discussion

*Biochemical and physiological characterization*

The results of phenotypic characterization using standard tests and API ZYM systems indicated that all of the strains isolated from seawater, diseased turbot and sole were rather homogeneous in their biochemical and physiological reactions and were very similar to the strains of *Tenacibaculum maritimum* used as reference in the study. All the strains were

Gram-negative, filamentous gliding bacteria that gave positive reactions for catalase, cytochrome oxidase, Congo red adsorption, nitrate reduction, gelatin and casein hydrolysis, and amylase. Negative reactions occurred for presence of flexirubin-type pigment, Indol, Voges Proskauer, acid production from carbohydrates, production of $SH_2$ and growth in Tryptic soya broth supplemented with 1% NaCl. The existence of phenotypic homogeneity within the species *T. maritimum* as well as within other species of the group *Cytophaga-Flavobacterium-Flexibacter* has been previously reported (Hikida et al, 1979; Wakabayashi et al., 1986; Pazos et al., 1993; Bernardet, 1997; Santos et al., 1999).

*PCR identification*

The PCR amplification of genomic DNA from *T. maritimum* reference strains using the oligonucleotide species-specific primers MAR1 and MAR2 described by Bader and Shotts (1998) generated a single product with the expected 400 bp length (data not shown). No amplification products were obtained from genomic DNA of *T. maritime*-like strains isolated from marine fish and seawater or from negative control reaction (no template). These results allow us to confirm that strains of filamentous gliding bacteria isolated from marine fish and seawater are not included in the species *T. maritimum*.

*Serological testing*

The results of slide agglutination assays demonstrated that, regardless of their biochemical similarities, the strains of *T. maritimum* and the strains of filamentous bacteria isolated from fish and seawater were antigenically different (Table 1). Serological heterogeneity was also observed within the *T. maritimum*-related organisms, with the typeable strains (9 of 23 strains tested) being distributed into four serological O-groups. Four strains isolated from seawater (A13.3, A35.1, A37.1 and A38.1) reacted with the serum anti-A37.1 and one strain (A52.1) reacted with the serum anti-R01.6.8.1. The strains R00.6.0.1, R01.6.8.1 and LL04.11.1.1 isolated from fish only reacted with their homologous antisera The existence of variability in serological characteristics has previously been reported in other bacteria isolated from marine fish culture systems such us *T. maritimum* (Pazos, 1997; Santos et al., 1999; Avendaño-Herrera et al., 2004), *Vibrio anguillarum* (Sørensen & Larsen, 1986; Austin et al., 1995; Santos et al., 1995), *Vibrio pelagius* and *V. splendidus* (Santos et al., 1996, 1997).

*Pathogenicity assays*

The virulence assays demonstrated that all the strains tested were pathogenic for turbot and sole and produced mortalities between 60 and 100% with bacterial doses ranging from $9.0 \times 10^5$ to $9.0 \times 10^7$ colony forming units (CFU) per fish. These lethal doses are similar to that previously described for *T. maritimum* strains (Magariños et al 1995; Pazos, 1997). The inoculated strains were reisolated in pure culture from the internal organs of all moribund and dead fish.

In conclusion, the strains of filamentous bacteria isolated from turbot and sole culture systems are antigenically and genetically different from *T. maritimum* species and can be serologically typed on the basis of their different "O" antigen. Further molecular and virulence studies are in progress in order to

Bull. Eur. Ass. Fish Pathol., 27(1) 2007, 34

clarify the taxonomic status of these filamentous organisms and to determine the real risk that *T. maritimum*-like micro-organisms represent for the culture of turbot and Senegalese sole.

## Acknowledgements

This investigation was supported by grant PGIDIT 04 RMA 003E from the Xunta de Galicia, Spain.

The authors acknowledge Stolt Sea Farm for the collaboration provided for the study.

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**Artículo nº2.** Piñeiro-Vidal, M. e Santos, Y. (2008). Caracterización serológica y molecular de una bacteria filamentosa aislada de lenguado (*Solea senegalensis*, Kaup). En "X Foro dos Recursos Mariños e da Acuicultura das Rías Galegas e I Foro Iberoamericano dos Recursos Mariños e da Acuicultura". pp. 567-575. Editores: Manuel Méndez Rey, Jacobo Fernández Casal, César Lodeiros Seijo y Alejandro Guerra Díaz. ISBN. 978-84-608-0755-1.

Foro Ac. Rec. Mar. Rías Gal. 10: 567-575 2008.

# Caracterización serológica y molecular de una bacteria filamentosa aislada de lenguado (*Solea senegalensis*, Kaup)

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**Palabras clave:** Flexibacteriosis, inmunoblot, RAPD, Tenacibaculum.

## Resumen

Este estudio describe la caracterización serológica y molecular de una bacteria filamentosa aislada a partir de lenguados enfermos con sintomatología típica de flexibacteriosis (colas erosionadas, hemorragias en la boca y lesiones epidérmicas) y que habían sido vacunados por baño con una vacuna comercial anti-*Tenacibaculum maritimum*. La caracterización fenotípica, serológica y molecular demostró que la cepa en estudio no pertenece a las especies *T. maritimum*, *T. gallaicum* o *T. discolor*. Los ensayos de patogenicidad indican que la bacteria aislada es virulenta para rodaballo y lenguado.

## Introducción

La flexibacteriosis marina es considerada como uno de los factores limitantes para el cultivo de especies de peces marinos de interés comercial en Europa, América y Asia (Santos *et al.,* 1999). Los signos externos característicos de la enfermedad son lesiones en la boca, úlceras en piel, erosión de aletas y cola (Bernardet *et al.,* 1990; Alsina and Blanch, 1993; Pazos *et al.,* 1993; Santos *et al.,* 1999). Aunque *T. maritimum* es considerado el principal agente causal de la enfermedad, en los últimos años se han descrito dentro del género *Tenacibaculum* otras especies aisladas a partir de huevos de halibut (*T. ovolyticum*, Hansen *et al.,* 1992), de lenguados enfermos (*T. discolor*, Piñeiro-Vidal *et al.,* 2008) y del agua de tanques de cultivo de rodaballo (*T. gallaicum*, Piñeiro-Vidal *et al.,* 2008). El presente trabajo describe la caracterización serológica y molecular de una bacteria filamentosa aislada a partir de lesiones externas de peces vacunados contra la flexibacteriosis causada por *T. maritimum*.

## Material y métodos

***Peces***

Lenguados enfermos (peso medio 5,2 g) con sintomatología típica de flexibacteriosis (colas erosionadas, hemorragias en la boca y lesiones epidérmicas) y que habían sido vacunados por baño con una vacuna comercial anti-*Tenacibaculum maritimum* y trasladados a la planta de cultivo donde se realizó el estudio una semana antes de la aparición de los síntomas.

***Aislamiento e identificación bacteriana***

Las muestras de riñón, hígado, bazo y lesiones externas de lenguados enfermos se sembraron en placas de agar de soja tripticaseina (Oxoid) suplementados con 1% de NaCl (TSA-1), Tiosulfato Citrato Bilis Sacarosa (TCBS) agar (Oxoid) y medio *Flexibacter maritimus* (FMM) agar (Pazos *et al.,* 1996) y se incubaron a 25ºC durante 48 horas. Los cultivos puros de las bacterias aisladas en los medios FMM y TSA-1 se identificaron usando métodos microbiológicos convencionales en tubo y placa y los sistemas comerciales API (API 20E and API ZYM, Biomerieux) (Bernardet *et al.,* 1990; Pazos *et al.,* 1993; Santos *et al.,* 1993). La sensibilidad a agentes antimicrobianos se evaluó por el método de difusión en agar (Barry & Thornsberry, 1991) utilizando los siguientes agentes antimicrobianos (micrograms/disco) suministrados por Oxoid: ampicilina (10), oxitetraciclina (30), trimetoprim-sulfametoxazol (23,7-1,2), norfloxacina (10), ácido fusídico (10), gentamicina (10), amoxicilina (30), enrofloxacina (5) and the vibriostatic agent O/129-Pteridine (150). Las placas se incubaron a 25ºC durante 48 horas. En todos los ensayos se incluyeron con fines comparativos cepas de referencia de *T. maritimum* (NCIMB 2153), *T. gallaicum* (DSM 18841) y *T. discolor* (DSM 18842).

La identificación serológica se realizó mediante los ensayos de aglutinación en portaobjetos y Dot blot como previamente se describió (Santos *et al.,* 1995; Piñeiro-Vidal *et al.,* 2007). En estos ensayos se emplearon como antígenos la célula completa inactivada con formol y el antígeno termoestable "O" y antisuero obtenido en conejo frente a las cepas de referencia de *T. maritimum* (NCIMB 2153), *T. gallaicum* (DSM 18841) y *T. discolor* (DSM 18842).

La identificación molecular de las bacterias filamentosas aisladas en el medio FMM se realizó mediante el análisis de los ácidos grasos y técnicas basadas en la reacción en cadena de la polimerasa (PCR) empleando cebadores específicos y arbitrarios. Para la extracción y análisis de los ácidos grasos se siguieron los protocolos descritos por el Microbial Identification System (MIDI, Microbial ID Inc.) (Sasser, 1990). Para la amplificación del ADN bacteriano se han empleando los cebadores específicos Mar-1 y Mar-2 descritos para *T. maritimum* (Bader & Shotts, 1998) y que amplifican un fragmento de 400 pares de bases del gen 16S rRNA y cebadores arbitrarios para la amplificación aleatoria del ADN (RAPD).

Para la amplificación del fragmento del gen 16S rRNA se utilizaron condiciones de amplificación descritos previamente (Cepeda & Santos, 2002). Para el análisis RAPD se empleó el sistema comercial Ready to Go RAPD analysis kit, (Healthcare, GE, UK) y las condiciones de amplificación sugeridas por el fabricante. Los fragmentos amplificados se analizaron en geles de agarosa con bromuro de etidio.

***Ensayos de patogenicidad***

Con el fin de determinar si los microorganismos aislados representan un peligro potencial para los cultivos de lenguado se procedió a la realización de infecciones experimentales. La cepa cultivada en FMM se resuspendió en solución salina a una concentración aproximada de $10^9$ céls/ml (tubo 3 escala MacFarland). Las infecciones experimentales se realizaron en lenguado y rodaballo (peso medio 15 g), mantenidos en tanques de 100 litros de agua de mar a 18ºC y con aireación. Los peces se inyectaron intracelómicamente con 0,1 ml de la suspensión bacteriana. Las mortalidades se contabilizaron diariamente por un período de 21 días y se consideraron causadas por el microorganismo inoculado cuando éste se recuperaba en cultivo puro a partir de órganos internos.

## Resultados y conclusión

A partir de órganos internos se aislaron bacterias Gram negativas que fueron identificados como *Vibrio splendidus* biotipo I y *T. maritimum*. De las heridas externas se aislaron bacterias filamentosas en cultivo puro o en cultivo mixto con bacilos Gram-negativos pertenecientes al género *Pseudomonas*. La caracterización fenotípica demostró que las bacterias filamentosas eran Gram-negativas, con movilidad deslizante, que producían catalasa y citocromo oxidasa y reducían nitratos pero no producían $SH_2$ ni presentaban pigmento del tipo flexirubina (Tabla I). Algunos aislados presentaron un perfil enzimático similar al obtenido con la cepa de referencia de *T. maritimum* NCIMB 2153 en las galerías API ZYM y fueron identificados como *T. maritimum* con el suero anti-NCIMB 2153 y los cebadores específicos. Una de las bacterias filamentosas aisladas carecía de actividad hemolítica frente a eritrocitos humanos y de rodaballo y mostraba un perfil API ZYM caracterizado por la ausencia de los enzimas tripsina y alfa-quimiotripsina, caracteres que si están presentes en las cepas de referencia de *T. maritimum, T. gallaicum* y *T. discolor* utilizadas en el estudio. El análisis serológico demostró que esta cepa no pertenecía a las especies *T. maritimum*, *T. gallaicum* y *T. discolor*. Este aislado presentaba un patrón de sensibilidad a antimicrobianos ligeramente diferente al obtenido con las cepas de referencia, siendo sensible a ácido fusídico y pteridina y resistente a norfloxacina, oxitetraciclina, trimetoprim-sulfametoxazol, gentamicina, amoxicilina y enrofloxacina.

**Tabla I.-** Características fenotípicas de las cepas de referencia de *T. maritimum*, *T. gallaicum* y *T. discolor* y de la bacteria filamentosa aislada en el presente estudio (cepa LL04 12.1.7).

| | Cepa en estudio | *T. mariritmum NCIMB 2153* | *T. gallaicum* DSM18841 | *T. discolor* DSM 18842 |
|---|---|---|---|---|
| Gram | - | - | - | - |
| Catalasa | + | + | + | + |
| Oxidasa | + | + | + | + |
| Movilidad | + | + | + | + |
| Reducción de nitratos | + | + | + | + |
| Rango de T (ºC) | 14-30 | 14-38 | 14-38 | 14-38 |
| Rango de pH | 6.0-8.0 | 6.0-8.0 | 6.0-8.0 | 6.0-8.0 |
| Hidrólisis de | | | | |
| Gelatina | + | + | + | + |
| Almidón | - | + | - | - |
| Tween 80 | - | + | - | - |
| Utilización de fuentes de Carbono | | | | |
| D (+)-Galactosa | - | - | - | - |
| D (+)-Glucosa | - | - | - | - |
| L-Prolina | - | - | + | + |
| L-Tirosina | - | - | - | - |
| APIZYM | | | | |
| Esterasa Lipasa (C 8) | + | + | + | + |
| Tripsina | - | + | + | + |
| a-Quimiotripsina | - | + | + | + |

El análisis mediante cromatografía de gases puso de manifiesto que las cepas de *T. maritimum*, *T. gallaicum* y *T. discolor* presentan perfiles de ácidos grasos distintos (Tabla II, Fig. 1). Además, la composición de ácidos grasos de estas tres especies difiere claramente del encontrado en la cepa filamentosa aislada en este trabajo que se caracteriza por el alto contenido en ácidos grasos insaturados (Tabla II, Fig. 1).

**Tabla II**.- Perfil de ácidos grasos de la cepa LL04 12.1.7 y las especies de *Tenacibaculum* patógenas para peces *T. maritimum*, *T. discolor*, *T. gallaicum*.

[a] Los ácidos grasos con valor medio inferior al 1% no se incluyen.

[b] Mezcla 3 contiene $C_{16:1}$ω7c/Iso$C_{15:0}$ 2OH

| **Acido graso[a]** | **Composición %** | | | |
|---|---|---|---|---|
| | ***T. maritimum*** **NCIMB2153** | ***T. discolor*** **DSM 18842** | ***T. gallaicum*** **DSM18841** | ***Tenacibaculum* sp** **LL04 12.1.7** |
| **Saturados** | | | | |
| C15:0 | -- | 4,8 | -- | 4,8 |
| **Insaturados** | | | | |
| C15.1w6c | 6,1 | 3,2 | 3,9 | 12,2 |
| C17:1w6c | 0.9 | 1,7 | 2,2 | 1,7 |
| **Cadenas ramificadas** | | | | |
| Iso-$C_{14:0}$ | 0,9 | 1,2 | -- | 2,4 |
| Iso-$C_{15:0}$ | 18.1 | 17,1 | 19,6 | 23,2 |
| IsoG-$C_{15:1}$ | 12,7 | 8,5 | 8,3 | 5,6 |
| Iso-$C_{16:0}$ | 0,6 | 3,6 | 3,5 | 1,7 |
| Iso-$C_{16:1}$ H | 2.3 | 3,8 | 1,9 | 2,4 |
| Iso$C_{17:1}$w9c | 1.2 | 1,3 | 0,8 | - |
| ante-iso-$C_{15:0}$ | 1.6 | 0,8 | 1.6 | 0,8 |
| **Hidroxilados** | | | | |
| $C_{15:0}$ 3OH | 2,4 | 2,2 | 2,9 | 3,2 |
| Iso$C_{15:0}$ 3OH | 13,9 | 4,8 | 6,5 | 10,6 |
| $C_{16:0}$ 3OH | 1,1 | 0,9 | 1,5 | 2,2 |
| Iso$C_{16:0}$ 3OH | 6,8 | 11,9 | 10,1 | 8,3 |
| Iso$C_{17:0}$ 3OH | 8,3 | 6,9 | 10,6 | 2,9 |
| Mezcla 3 | 15,3 | 18,3 | 17,8 | 16,8 |

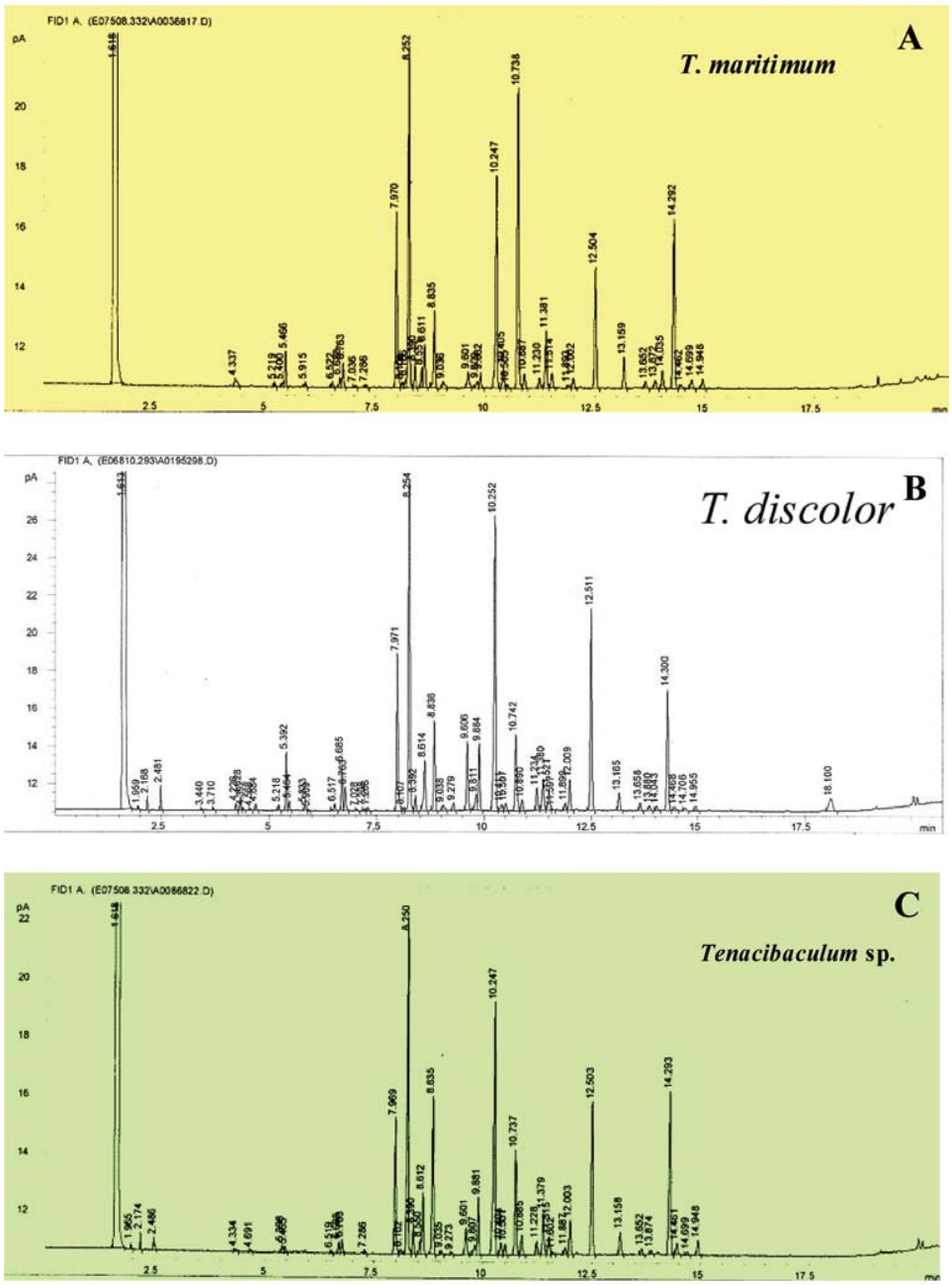


**Figura 1**.- Perfil de ácidos grasos de *T. maritimum* (A), *T. discolor* (B) y *Tenacibaculum* sp. (C).

Los perfiles de RAPD obtenidos con los cebadores 1 y 2 mostraron que la cepa filamentosa en estudio es genéticamente diferente de las especies *T. maritimum*, *T. gallaicum* y *T. discolor* (datos no mostrados).

Las características fenotípicas, serológicas y moleculares obtenidas en el presente estudio junto con el análisis de las secuencias del gen 16S rRNA y del contenido en G+C nos permitirán en un futuro próximo establecer si la bacteria filamentosa aislada constituye una nueva especie dentro del género *Tenacibaculum*.

## Agradecimientos

Esta investigación fue subvencionada parcialmente por los proyectos PGIDIT 04 RMA 003E and PGIDIT 06 RMA 024E de la Xunta de Galicia.

Los autores agradecen a Stolt Sea Farm la colaboración prestada en la realización del presente trabajo.

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# Caracterización serológica y molecular de una bacteria filamentosa aislada de lenguado (*Solea senegalensis*, Kaup.)

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**Introducción:**

La flexibacteriosis marina es actualmente la mayor causa de mortalidad en cultivos de rodaballo (*Psetta maxima*, L.) y lenguado (*Solea senegalensis*, Kaup.) en la comunidad gallega. La patología es causada por las bacterias filamentosas *Tenacibaculum maritimum*, *T. gallaicum* y *T. discolor* (Piñeiro-Vidal *y col.*, 2007 a). Recientemente nuestro grupo aisló a partir de lenguados enfermos, una bacteria filamentosa que identificamos de forma presuntiva como miembro del género *Tenacibaculum*. El objetivo del estudio ha sido la caracterización serológica y molecular del aislado.

## Material y métodos

**a) Cepas bacterianas:** Se han utilizado cepas de *T. maritimum* (NCIMB 2153, y LL01.8.3.8), *T. discolor* (DSM18842) y *T. gallaicum* (DSM 18841, RO 06.0.1) y la cepa de *Tenacibaculum* sp. asislada de lenguado (LL04 12.1.7). Las cepas se cultivaron en medio sólido FMM a 25°C durante 48h y se conservaron en viales de congelación Microbank (Ontario, Canadá).

**b) Caracterización taxonómica:** Para la identificación de los aislados se han utilizado pruebas morfológicas, fisiológicas y bioquímicas utilizando métodos microbiológicos convencionales y el sistema API ZYM y métodos serológicos (aglutinación en portaobjetos) utilizando sueros anti-*T. maritimum* NCIMB2153, anti-*T. discolor* DSM18842 y anti-*T. gallaicum* DSM 18841 y anti-LL04 12.1.7 como se describió previamente (Pazos, 1997; Piñeiro-Vidal et al., 2007 b).

**c) Análisis de ácidos grasos:** El análisis de ácidos grasos se realizó por cromatografía de gases según el protocolo MIDI.

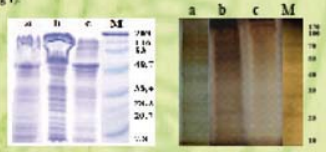
**d) Análisis electroforético e inmunológico de proteínas y LPS:** Las proteínas totales (PT) y lipopolisacáridos (LPS) se separaron por electroforesis en geles de poliacrilamida-SDS. Las proteínas se tiñeron con azul de Comassie y los LPS con la tinción de nitrato de plata. En el ensayo inmunológico (Dot blot) se utilizaron como antígenos LPS y proteínas de membrana de las cepas en estudio y los sueros anti-*T. maritimum* NCIMB2153, anti-*T. discolor* DSM18842, anti-*T. gallaicum* DSM 18841 y anti-LL04 12.1.7. El ensayo se realizó según la metodología descrita por Pazos, 1997).

**e) Amplificación aleatoria del ADN (RAPD) :** El ADN se extrajo con el sistema comercial Instagene (Biorad). Para la amplificación aleatoria del DNA se utilizó el sistema Ready-To-Go™ RAPD analysis Kit analysis (GE Healthcare, UK). Los fragmentos amplificados se analizaron en geles de agarosa, siguiendo las recomendaciones de la casa comercial.

## Resultados y conclusiones

* Las pruebas taxonómicas y el ensayo de aglutinación en portaobjetos indican que la cepa LL04 12.1.7 no pertenece a las especies *T. maritimum*, *T. gallaicum* y *T. discolor*.

* La cepa LL04 12.1.7 presenta un perfil de proteínas y LPS diferente al observado en las cepas tipo de las especies *T. gallaicum* o *T. discolor* (Fig 1).




* El ensayo dot-blot corroboran los resultados taxonómicos. Además, aunque existe cierta relación inmunológica entre las proteínas de membrana de las cepas en estudio, el antígeno lipopolisacarídico de la cepa LL04 12.1.7 y de las especies patógenas de peces *T. maritimum*, *T. gallaicum* o *T. discolor* son inmunológicamente diferentes (Fig 2).


**Figura 2.-** Dot-blot empleando como antígenos PT (A) y LPS (B) de las cepas en estudio y el suero anti-LL04 12.1.7. 2a, cepa LL04 12.1.7; 3a, *T. maritimum* LL01 8.3.8; 1b, *T. maritimum* NCIMB 2158; 2b, *T. gallaicum* DSM 18841$^T$; 2c, *T. gallaicum* RO06.0.1; 3a, *T. discolor* DSM 18842$^T$.

* La cepa LL04 12.1.7 presenta un perfil de RAPD similar al observado en las cepas de referencia de las especies *T. maritimum*, *T. galaicum* o *T. discolor*.

## Referencias

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**Piñeiro-Vidal, M., Riaza, A. and Santos, Y. (2007a)** *Tenacibaculum discolor* sp. nov. and *Tenacibaculum gallaicum* sp. nov. isolated from sole (*Solea senegalensis*) and turbot (*Psetta maxima*) culture systems. *Int J Syst Evol Microbiol* (in press).

**Piñeiro-Vidal, M., Carezzo-Sestelo, C., Riaza, A., and Santos, Y. (2007 b)** Isolation of pathogenic *Tenacibaculum maritimum*-related organisms from diseased turbot and sole cultured in the Northwest of Spain. *Bull Eur Ass Fish Pathol.* 27, 29-35.

# III B. Láminas complementarias

## LÁMINAS COMPLEMENTARIAS

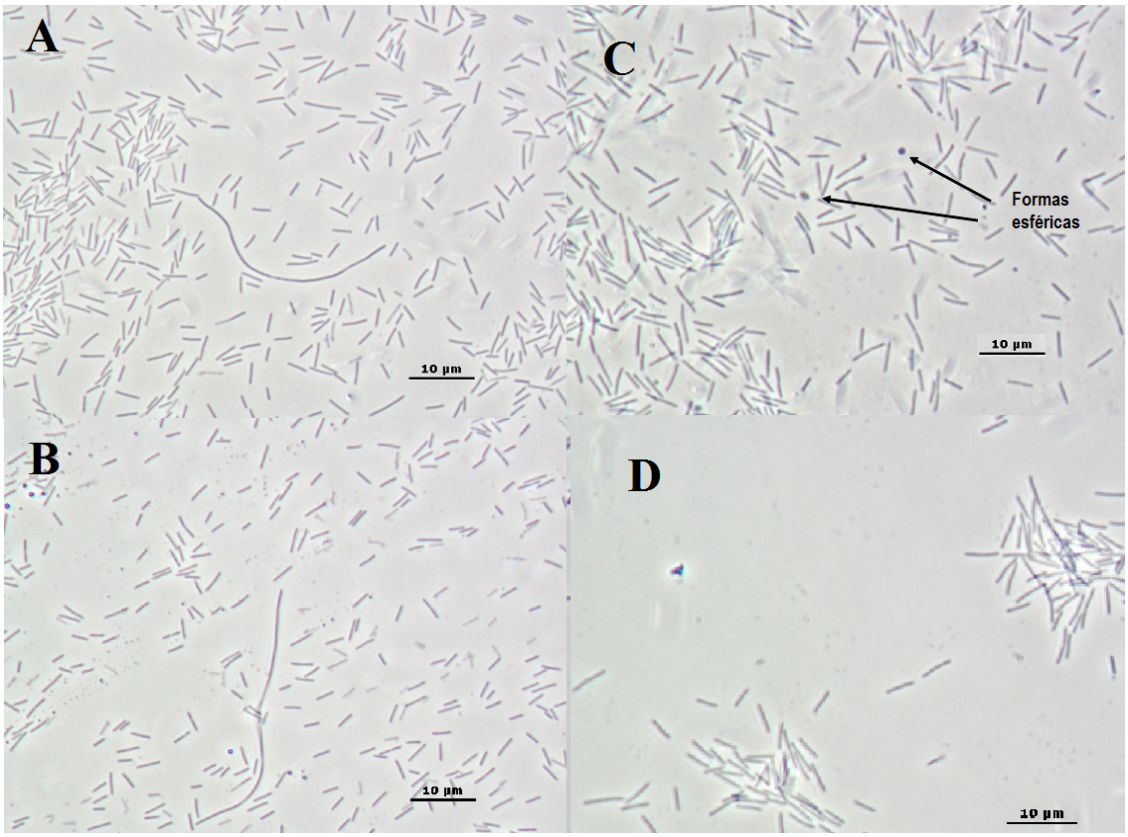


Lámina 1.- Morfología al microscopio óptico (1000X) de los microorganismos en estudio A, Cepa A37.1; B, Cepa LL04 11.1.1; C, *T. maritimum*, NCIMB 2154; D,. LL04 12.1.7

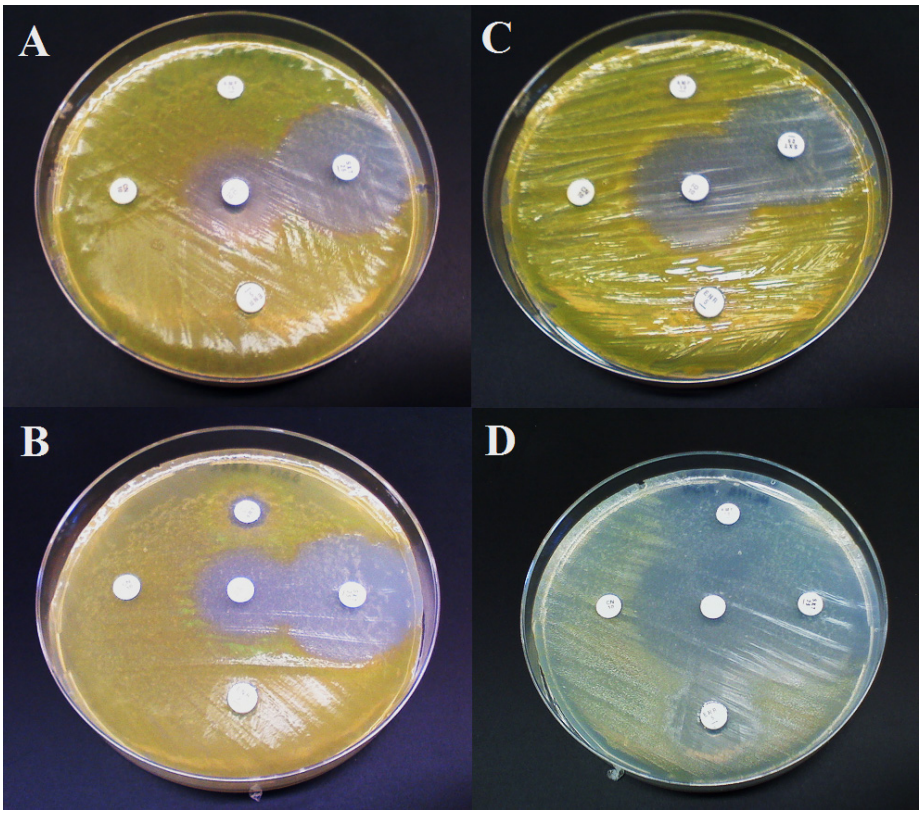


Lámina 2.- Ensayo de sensibilidad a agentes antimicrobianos de cepas de *Tenacibaculum* utilizadas en el estudio. A, Cepa A37.1; B, Cepa LL04 11.1.1; C,LL04 12.1.7; D, *T. maritimum*, NCIMB 2154.

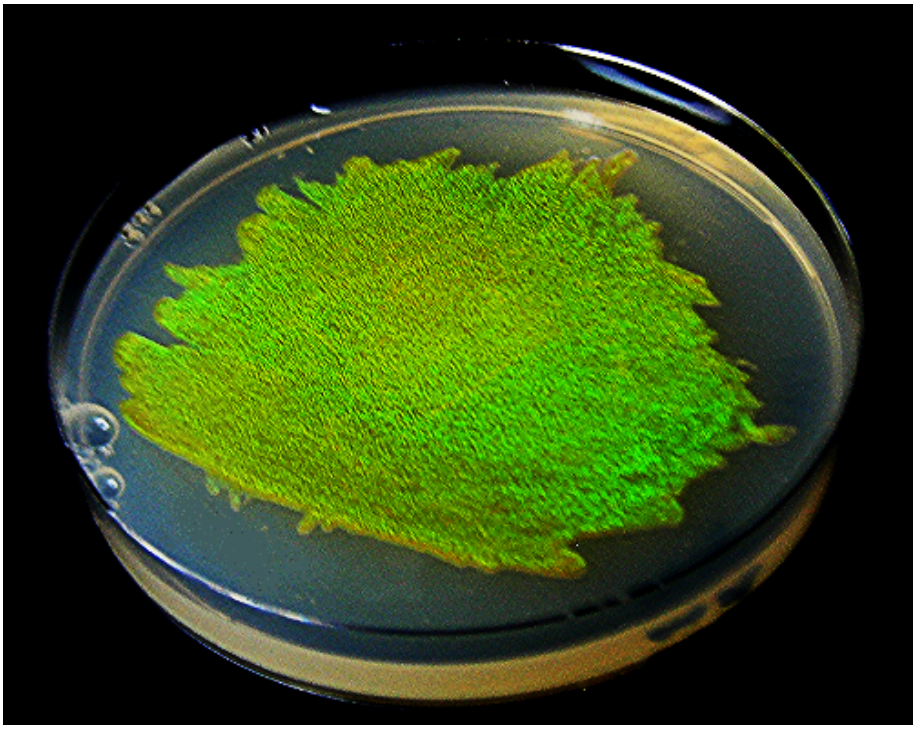

Lámina 3.- Aspecto de un cultivo de 24 h en agar marino de la cepa LL04 11.1.1.

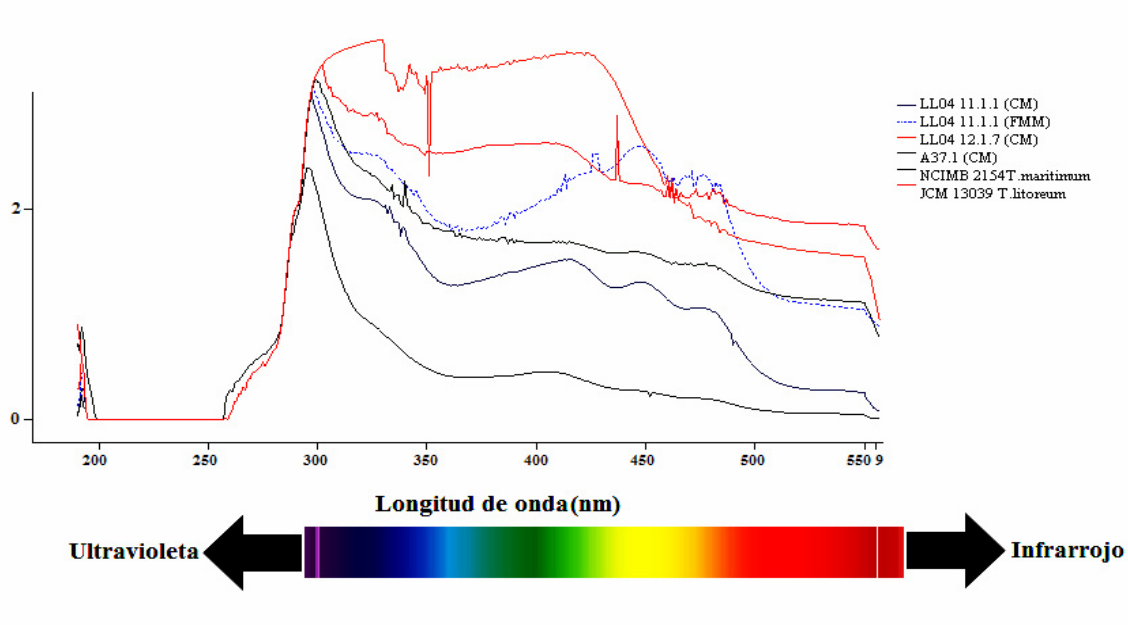


Lámina 4.- Espectro de absorción de los pigmentos extraídos de las cepas de las especies en estudio *T. maritimum,* A37.1, LL04 11.1.1 y LL0412.1.7.

# Capítulo IV. Análisis molecular de bacterias filamentosas aisladas de sistemas de cultivo de rodaballo y lenguado: descripción de tres nuevas especies del género *Tenacibaculum*.

# Capítulo IV A. Publicaciones

**Artículo nº3**. Piñeiro-Vidal, M., Riaza, A., Santos, Y. (2008). *Tenacibaculum discolor* sp. nov. and *Tenacibaculum gallaicum* sp. nov. isolated from sole (*Solea senegalensis*) and turbot (*Psetta maxima*) culture systems. *International Journal of Systematic and Evolutionary Microbiology* **58**, 21-25.

*International Journal of Systematic and Evolutionary Microbiology* (2008), 58, 21–25 DOI 10.1099/ijs.0.65397-0

# *Tenacibaculum discolor* sp. nov. and *Tenacibaculum gallaicum* sp. nov., isolated from sole (*Solea senegalensis*) and turbot (*Psetta maxima*) culture systems

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Two Gram-negative, rod-shaped, gliding bacterial strains, designated strain LL04 11.1.1$^T$ and strain A37.1$^T$, were isolated from a diseased sole (*Solea senegalensis*) and from seawater from a holding tank for turbot (*Psetta maxima*), respectively. The strains grew on solid media as bright yellow colonies with uneven edges; the colonies did not adhere to the agar. The bacteria were able to grow at temperatures in the range 14 to 38 °C and from pH 6.0 to pH 8.0. The DNA G+C contents of strains LL04 11.1.1$^T$ and A37.1$^T$ were 32.1 and 32.7 mol%, respectively. Analysis of the 16S rRNA gene sequences indicated that strains LL04 11.1.1$^T$ and A37.1$^T$ were members of the genus *Tenacibaculum* in the family *Flavobacteriaceae*. The sequence similarities of the two isolates with respect to the type strains of recognized members of the genus ranged from 94.2 to 99.4 %. DNA–DNA hybridization studies revealed that the strains studied represent two distinct novel species of the genus *Tenacibaculum*, for which the names *Tenacibaculum discolor* sp. nov. [type strain LL04 11.1.1$^T$ (=NCIMB 14278$^T$=DSM 18842$^T$)] and *Tenacibaculum gallaicum* sp. nov. [type strain A37.1$^T$ (=NCIMB 14147$^T$=DSM 18841$^T$)] are proposed.

The genus *Tenacibaculum*, proposed by Suzuki *et al.* (2001), belongs to the family *Flavobacteriaceae* (Reichenbach, 1992a, b; Bernardet *et al.*, 1996, 2002) and currently comprises nine species: *Tenacibaculum maritimum* from diseased fish (Wakabayashi *et al.*, 1986), *T. ovolyticum* from fish eggs (Hansen *et al.*, 1992), *T. amylolyticum* from the surfaces of marine macroalgae and *T. mesophilum* from sponges (Suzuki *et al.*, 2001), *T. skagerrakense* isolated from seawater (Frette *et al.*, 2004), *T. lutimaris*, *T. litoreum*, *T. aestuarii* from tidal flat sediment (Yoon *et al.*, 2005; Choi *et al.*, 2006; Jung *et al.*, 2006) and *T. litopenaei* isolated from a shrimp mariculture pond (Sheu *et al.*, 2007).

During the characterization of bacteria isolated from the kidney of a diseased sole (*Solea senegalensis*) and from seawater from holding tanks for turbot (*Psetta maxima*), strains LL04 11.1.1$^T$ and A37.1$^T$ were recovered on plates of *Flexibacter maritimus* medium (FMM; Pazos *et al.*, 1996) at 25 °C (*T. maritimum* was previously misclassified within the genus *Flexibacter*). The diseased fish showed the typical signs observed in fish affected by marine flexibacteriosis caused by *T. maritimum* (i.e. eroded mouth, rotten fins, shallow skin lesions and paleness of internal organs). Pathogenicity assays demonstrated that the isolated bacteria were virulent for turbot and sole (Piñeiro-Vidal *et al.*, 2007). The isolated bacteria were subcultured on FMM agar at 25 °C for 48 h and maintained at −80 °C both in FMM broth supplemented with 15 % (v/v) glycerol and in Microbank tubes (Prolab Diagnostics).

Morphological, physiological and biochemical tests were performed as described by Bernardet *et al.* (2002). The Gram reaction was tested by using the bioMérieux Gram-stain kit (according to the manufacturer's instructions) and the non-staining KOH method (Buck, 1982). Gliding motility was determined by phase-contrast microscopic examination of a fresh FMM broth culture and by the hanging drop technique as recommended by Bernardet *et al.* (2002). The presence of flexirubin-type pigments was determined by using the KOH test as described by Reichenbach (1989). Catalase and oxidase activities were determined as described by Cowan & Steel (1965). The ability of the strains to grow under anaerobic conditions was tested in FMM agar by using the GasPak anaerobic system (BBL). To assess their growth at different pH values,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LL04 11.1.1$^T$ and A37.1$^T$ are AM411030 and AM746477, respectively.

the strains were cultured in FMM broth adjusted to pH values ranging from 4 to 10. The temperature range for growth was determined on FMM agar plates incubated at 8, 15, 18, 22, 25, 30, 37 and 44 °C. Tolerance of salinity was tested in FMM broth containing seawater strengths of 10, 20, 30, 50, 70 and 100 % or at NaCl concentrations of 0.8, 1, 3, 5, 7 and 10 % (w/v). Indole production was tested in FMM broth supplemented with 1 % (w/v) tryptone, $H_2S$ production was determined in FMM broth supplemented with 5 % (w/v) peptone and the Voges–Proskauer reaction was determined in seawater supplemented with 0.7 % (w/v) peptone and 0.5 % (w/v) D-glucose. The ability of the novel strains to degrade casein (1 %), gelatin (1 %), starch (0.4 %) and Tween 80 (1 %) and to produce nitrate reductase were evaluated in FMM medium supplemented with substrate concentrations as reported previously (Suzuki *et al.*, 2001). The utilization of carbon sources was tested on basal agar medium [containing, $l^{-1}$ artificial seawater (Sigma), 0.2 g $NaNO_3$, 0.2 g $NH_4Cl$, 0.05 g yeast extract, 15 g agar] supplemented with 0.4 % carbon source [(+)-D-sucrose, (−)-D-ribose, (+)-D-galactose, (+)-D-glucose, L-proline, L-glutamate or L-tyrosine] as described by Suzuki *et al.* (2001). The absence of growth after incubation in the media for 1 month was scored as a negative result. Other enzymic activities were evaluated using the API ZYM system (bioMérieux) according to the manufacturer's instructions, except that sterile seawater was used as the suspension medium.

The results of the morphological, physiological and biochemical tests are given in the species descriptions and in Table 1.

Sequencing of the 16S rRNA gene of the isolates was carried out by the Identification Service of the Colección Española de Cultivos Tipo (Universidad de Valencia, Valencia, Spain). The resulting sequences of strains LL04 11.1.1$^T$ (1512 bp) and A37.1$^T$ (1518 bp) were aligned automatically (using CLUSTAL W; Thompson *et al.*, 1994) with those of the type strains of the nine species of the genus *Tenacibaculum* and other representative members of the family *Flavobacteriaceae* obtained from GenBank. The 16S rRNA gene sequence similarities were determined using MEGA, version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods (Fig. 1). The evolutionary distance matrix for the neighbour-joining method was generated according to the Kimura two-parameter model (Kimura, 1980). The robustness of the phylogenetic trees was determined by means of bootstrap analyses based on 1000 replications. In both trees, strains LL04 11.1.1$^T$ and A37.1$^T$ formed a robust cluster with the species of the genus *Tenacibaculum*. Data from the sequence similarity analysis indicated that the closest relatives of strains LL04 11.1.1$^T$ and A37.1$^T$ were *T. litoreum* (99.4 and 98.4 %, respectively), *T. mesophilum* (97.4 and 96.7 %, respectively), *T. aestuarii* (97.3 % for both isolates), *T. lutimaris* (97.3 and 96.8 %, respectively), *T. skagerrakense* (96.4 and 95.9 %, respectively), and *T. amylolyticum* and (96.0 and 96.3 %, respectively). The 16S rRNA gene sequence similarity between strains LL04 11.1.1$^T$ and A37.1$^T$ was 98.4 %.

The DNA G+C content was determined using the method described by Cashion *et al.* (1977). As the 16S rRNA gene sequence similarities between strains LL04 11.1.1$^T$ and A37.1$^T$ and between them and the type strain of *T. litoreum* were close to 99 %, DNA–DNA hybridization experiments were performed using the method described by De Ley *et al.* (1970), as modified by Huß *et al.* (1983). The experiment was performed at 45 °C in 2 × SSC (1 × SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate at pH 7.0) using a Cary 100 Bio UV/visible spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). The DNA G+C content determinations and the DNA–DNA hybridization experiments were performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The DNA G+C contents of strains LL04 11.1.1$^T$ and A37.1$^T$ were 32.1 and 32.7 mol%, respectively (Table 1). The DNA–DNA relatedness between strains LL04 11.1.1$^T$ and A37.1$^T$ was 42.8 %, and the strains shared 39.6 and 40.2 % relatedness, respectively, with their closest relative, *T. litoreum*. Therefore, we conclude that strains LL04 11.1.1$^T$ and A37.1$^T$ represent two novel bacterial species within the genus *Tenacibaculum*, for which we propose the names *Tenacibaculum discolor* sp. nov. and *Tenacibaculum gallaicum* sp. nov., respectively.

### Description of *Tenacibaculum discolor* sp. nov.

*Tenacibaculum discolor* (dis.co'lor. L. neut. adj. *discolor* of different colours, referring to the colours of the colonies).

Cells are Gram-negative rods that are 0.5 μm in diameter, 2–30 μm in length and motile by gliding. Spherical cells are rarely observed in ageing cultures. Colonies on FMM agar medium are flat, bright yellow, have uneven edges and do not adhere to the agar. On marine agar 2216 (Difco), colonies also appear bright yellow from the front, but appear bright green when viewed at an angle of 30–45 °C. The yellow pigment is not of the flexirubin type. Strictly aerobic. Growth occurs in media containing seawater at strengths in the range 30 to 100 % but not in media supplemented with NaCl alone. Growth occurs at 14–38 °C (optimum, 25–30 °C) and at pH 6.0–8.0. Catalase and cytochrome oxidase activities are present. Nitrate is reduced. Gelatin and casein are hydrolysed, but Tween 80 and starch are not. The Voges–Proskauer test gives a negative result. No acid is produced from carbohydrates. $H_2S$ and indole are not produced. L-Proline and L-glutamate are utilized, but (+)-D-sucrose, (−)-D-ribose, (+)-D-galactose, (+)-D-glucose and L-tyrosine are not utilized. In the API ZYM system, alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin,

**Table 1.** Differential phenotypic characteristics of strains LL04 11.1.1$^T$ and A37.1$^T$ and the type strains of species of the genus *Tenacibaculum*

Taxa: 1, strain LL04 11.1.1$^T$; 2, strain A37.1$^T$; 3, *T. mesophilum* MBIC1140$^T$; 4, *T. lutimaris* KCTC 12302$^T$; 5, *T. skagerrakense* ATCC BAA-458$^T$; 6, *T. amylolyticum* MBIC4355$^T$; 7, *T. ovolyticum* IAM 14318$^T$; 8, *T. maritimum* NCIMB 2154$^T$, 9, *T. litoreum* JCM 13039$^T$; 10, *T. aestuarii* KCTC 12569$^T$; 11, *T. litopenaei* BCRC 17590$^T$. Data are from Wakabayashi *et al.* (1986), Hansen *et al.* (1992), Suzuki *et al.* (2001), Bernardet *et al.* (2002), Frette *et al.* (2004), Yoon *et al.* (2005), Choi *et al.* (2006), Jung *et al.* (2006), Sheu *et al.* (2007) and this study. +, Positive; −, negative; W, weakly positive; NT, not tested; NG, no growth.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Origin | Diseased sole | Seawater from holding tanks for turbot | Sponges, macroalgae | Tidal flat sediment | Seawater | Macroalgae | Halibut eggs | Diseased marine fish | Tidal flat sediment | Tidal flat sediment | Shrimp mariculture pond |
| Cell size (μm) | 2–30 × 0.5 | 2–30 × 0.5 | 1.5–10 × 0.5 | 2–10 × 0.5 | 2–15 × 0.5 | 2–5 × 0.4 | 2–20 × 0.5 | 2–30 × 0.5 | 2–35 × 0.3–0.5 | 2.0–3.5 × 0.3 | 2–10 × 0.3–0.5 |
| Gliding motility | + | + | + | + | − | + | + | + | + | + | + |
| NaCl concentration (%) for growth | NG | NG | 1–7 | <8 | NG | 3 | NG | NG | 3–5 | <7 | 2–10 |
| Seawater strength (%) for growth | 30–100 | 30–100 | 10–100 | 25–175 | 25–150 | 50–100 | 70–100 | 30–100 | 25–250 | NT | NT |
| Temperature range (°C) | 14–38 | 14–38 | 15–40 | 10–39 | 10–40 | 20–35 | 4–25 | 15–34 | 5–40 | 9–41 | 10–39 |
| Optimal temperature (°C) | 25–30 | 25–30 | 28–35 | 30–37 | 25–37 | 27–30 | NT | 25–30 | 35–40 | 30–37 | 28–37 |
| pH range | 6.0–8.0 | 6.0–8.0 | 5.3–9 | 7.0–8.0 | 6.0–9.0 | 5.3–8.3 | 5.9–8.6 | 5.9–8.6 | 6.0–10.0 | 5.5–8.5 | 5–10 |
| Nitrate reduction | + | + | − | − | + | W | + | + | + | − | − |
| Degradation of: | | | | | | | | | | | |
| Starch | − | − | − | − | + | + | − | − | + | − | + |
| Gelatin | + | + | + | + | NT | + | + | + | + | + | + |
| Tween 80 | − | − | + | − | − | + | + | + | + | + | − |
| Carbon utilization: | | | | | | | | | | | |
| (+)-D-Sucrose | − | − | − | − | + | − | − | − | − | − | − |
| (−)-D-Ribose | − | − | − | NT | NT | − | − | − | − | − | NT |
| (+)-D-Galactose | − | − | NT | NT | NT | NT | NT | − | − | − | − |
| (+)-D-Glucose | − | − | NT | − | + | NT | NT | − | − | − | + |
| L-Proline | + | + | + | − | + | + | − | − | + | − | + |
| L-Glutamate | + | + | + | − | + | + | − | W | − | − | + |
| L-Tyrosine | − | − | NT | NT | NT | NT | NT | − | NT | NT | NT |
| DNA G+C content (mol%) | 32.1 | 32.7 | 31.6–32.0 | 32.3–32.8 | 35.2 | 31.6–32.0 | 30.3–32.0 | 31.3–32.5 | 30 | 33.6 | 35.2 |

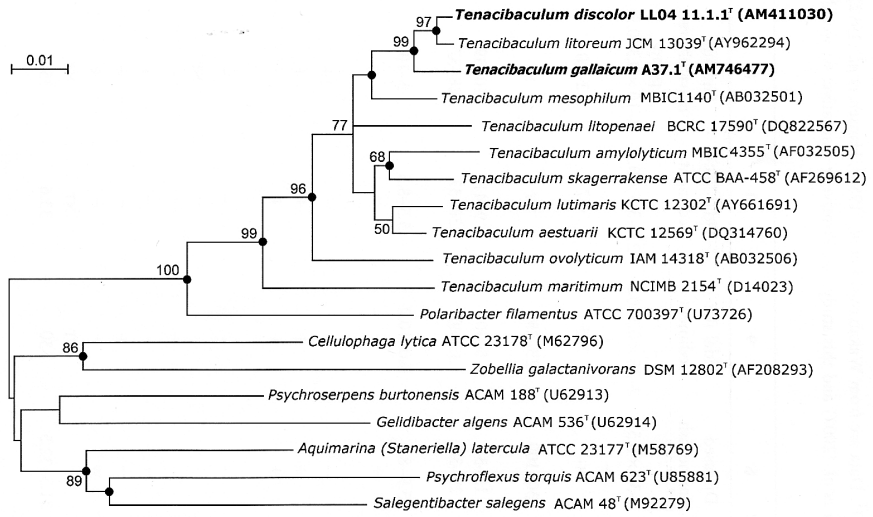


**Fig. 1.** Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains LL04 11.1.1$^T$ and A37.1$^T$ and members of the genus *Tenacibaculum* and other related genera belonging to the family *Flavobacteriaceae*. Bootstrap percentages (based on 1000 replications) are shown at the branching points. Filled circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Bar, 0.01 nucleotide substitutions per site.

acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but activities for all of the enzymes relating to the metabolism of carbohydrates are absent.

The type strain, LL04 11.1.1$^T$ (=NCIMB 14278$^T$=DSM 18842$^T$), was isolated from the kidney of a diseased sole (*Solea senegalensis*) in Galicia in north-west Spain.

### Description of *Tenacibaculum gallaicum* sp. nov.

*Tenacibaculum gallaicum* (gal.lai'cum. L. neut. adj. *gallaicum* of Galicia, a north-western province of Spain, referring to the place of isolation).

Cells are Gram-negative rods that are 0.5 μm in diameter, 2–30 μm in length and motile by gliding. Spherical cells are rarely observed in ageing cultures. Colonies on FMM agar and marine agar 2216 media are flat, bright yellow, have uneven edges and do not adhere to the agar. Strictly aerobic. Growth occurs in media containing seawater strengths of 30–100 %, but not in media supplemented with NaCl alone. Growth occurs at 14–38 °C (optimum, 25–30 °C) and at pH 6.0–8.0. Catalase and cytochrome oxidase activities are present. Nitrate is reduced. Gelatin and casein are hydrolysed, but Tween 80 and starch are not hydrolysed. The Voges–Proskauer test gives a negative result. No acid is produced from carbohydrates. $H_2S$ and indole are not produced. L-Proline and L-glutamate are utilized but (+)-D-sucrose, (−)-D-ribose, (+)-D-galactose, (+)-D-glucose and L-tyrosine are not utilized. In the API ZYM system, alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but all of the enzymes relating to the metabolism of carbohydrates are absent.

The type strain, A37.1$^T$ (=NCIMB 14147$^T$=DSM 18841$^T$), was isolated from seawater from a holding tank for turbot (*Psetta maxima*), in Galicia in north-west Spain.

## Acknowledgements

This investigation was supported by grant PGIDIT 04 RMA 003E from the Xunta de Galicia, Spain. The authors acknowledge Stolt Sea Farm for collaboration during this study, Dr J. P. Euzéby (Laboratoire de Bactériologie, École Nationale Vétérinaire, Toulouse, France) for his help with species names, and the referees for constructive suggestions.

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**Artículo nº4**. Piñeiro-Vidal, M., Carballas, C. G., Gómez-Barreiro, O., Riaza, A., & Santos, Y. (2008). *Tenacibaculum soleae* sp. nov. isolated from diseased sole (*Solea senegalensis*, Kaup). *International Journal of Systematic and Evolutionary Microbiology* **58**, 881-885.

*International Journal of Systematic and Evolutionary Microbiology* (2008), **58**, 881–885 DOI 10.1099/ijs.0.65539-0

# *Tenacibaculum soleae* sp. nov., isolated from diseased sole (*Solea senegalensis* Kaup)

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A novel Gram-negative, rod-shaped, gliding bacterial strain designated LL04 12.1.7$^T$ was isolated from diseased sole (*Solea senegalensis* Kaup) in Galicia, Spain. Colonies were yellow-pigmented with uneven edges and did not adhere to the agar. The DNA G+C content of the strain was 29.8 mol%. 16S rRNA gene sequence similarity analysis indicated that strain LL04 12.1.7$^T$ is a member of the genus *Tenacibaculum* in the family *Flavobacteriaceae*. Sequence similarities between the isolate and the type strains of other members of the genus were 96.7–94.8 %. The major fatty acids (>10 % of total fatty acids) were iso-$C_{15:0}$ (23.1 %), iso-$C_{15:0}$ 3-OH (10.6 %), $C_{15:1}\omega6c$ (12.2 %) and summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, 11.0 %). Genotypic and phenotypic data distinguished strain LL04 12.1.7$^T$ from the 11 recognized *Tenacibaculum* species, indicating that it represents a novel species, for which the name *Tenacibaculum soleae* sp. nov. is proposed. The type strain is strain LL04 12.1.7$^T$ (=CECT 7292$^T$ =NCIMB 14368$^T$).

The genus *Tenacibaculum* (Suzuki *et al.*, 2001) in the family *Flavobacteriaceae* (Reichenbach, 1992a, b; Bernardet *et al.*, 1996, 2002) currently comprises 11 species derived from different marine ecosystems and intensive aquaculture systems. *Tenacibaculum maritimum* and *Tenacibaculum discolor* were isolated from diseased fish (Wakabayashi *et al.*, 1986; Piñeiro-Vidal *et al.*, 2008); *Tenacibaculum ovolyticum* was isolated from fish eggs (Hansen *et al.*, 1992); *Tenacibaculum litopenaei* (Sheu *et al.*, 2007) and *Tenacibaculum gallaicum* (Piñeiro-Vidal *et al.*, 2008) were from seawater of shrimp- and turbot-holding tanks, respectively; *Tenacibaculum amylolyticum* and *Tenacibaculum mesophilum* were from marine macroalgae and sponge, respectively (Suzuki *et al.*, 2001); *Tenacibaculum skagerrakense* was from seawater (Frette *et al.*, 2004); and *Tenacibaculum lutimaris* (Yoon *et al.*, 2005), *Tenacibaculum litoreum* (Choi *et al.*, 2006) and *Tenacibaculum aestuarii* (Jung *et al.*, 2006) were from tidal flat sediment.

During the characterization of bacteria isolated from a diseased cultured sole (*Solea senegalensis* Kaup), strain LL04 12.1.7$^T$ was recovered on plates of *Flexibacter maritimus* medium (FMM) (Pazos *et al.*, 1996). Subcultivation was done on FMM or marine agar 2216 (MA; Difco) at 25 °C for 48 h. Strains were preserved at −80 °C in both marine broth 2216 (MB; Difco) supplemented with 15 % (v/v) glycerol and Microbank tubes (Prolab Diagnostics). Experimental infection assays have demonstrated that strain LL04 12.1.7$^T$ is virulent for fingerlings of sole and turbot, but not for mice (data not shown).

Morphological, physiological and biochemical tests were performed as described by Bernardet *et al.* (2002). The Gram reaction was tested by using the bioMérieux Gram stain kit according to the manufacturer's instructions and the non-staining KOH method (Buck, 1982). Gliding motility was determined by phase-contrast microscopic examination of a fresh MB culture by the hanging drop technique as recommended by Bernardet *et al.* (2002). The presence of flexirubin-type pigments was determined by using the KOH test as described by Reichenbach (1989). Catalase and oxidase activities were determined as described by Cowan & Steel (1965). The capacity of the strain to grow under anaerobic conditions was tested on MA using the GasPak anaerobic system (BBL). The optimal pH and the pH range for growth were determined in FMM broth adjusted to pH 4–10 according to Suzuki *et al.* (2001). Growth at various temperatures (8, 15, 18, 22, 25, 30, 37 and 44 °C) was determined on FMM agar plates. Tolerance to salinity was tested in FMM broth containing 10, 20, 30, 50, 70 or 100 % seawater or 0.8, 1, 3, 5, 7 or 10 % (w/v) NaCl. Indole and $H_2S$ production were tested on FMM broth supplemented with 1 % (w/v) tryptone or 5 %

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LL04 12.1.7$^T$ is AM746476.

(w/v) peptone, respectively. The Voges–Proskauer reaction was evaluated in seawater with 0.7 % (w/v) peptone and 0.5 % (w/v) (+)-D-glucose. The capacity of the strain to degrade casein (1 %), gelatin (1 %), starch (1 %) and Tween 80 (1 %) was evaluated in FMM medium (Suzuki *et al.*, 2001). Utilization of carbon sources was tested on basal agar medium (0.2 g $NaNO_3$, 0.2 g $NH_4Cl$, 0.05 g yeast extract and 15 g agar in 1 l artificial seawater) containing 0.4 % carbon source [sucrose, (−)-D-ribose, (+)-D-galactose, (+)-D-glucose, L-proline, L-glutamate or L-tyrosine] as described by Suzuki *et al.* (2001). The absence of growth after 1 month of incubation was scored as a negative result. Other enzyme activities were evaluated in the API ZYM system (bioMérieux) following the manufacturer's instructions, except that sterile seawater was used as the suspension medium.

For analysis of fatty acid methyl esters, strain LL04 12.1.7$^T$ was grown on MA plates for 48 h at 25 °C. Cell harvesting, saponification of lipids, methylation of fatty acids, extraction of fatty acid methyl esters, washing of extracts and GC analysis were performed according the standardized procedures of the Microbial Identification system (MIDI; Microbial ID) (Sasser, 1990).

Determination of the DNA G+C content and sequencing of the 16S rRNA gene of the isolate were carried out by the identification service of the DSMZ, Braunschweig, Germany. The 1506 bp sequence of strain LL04 12.1.7$^T$ was automatically aligned using CLUSTAL W (Thompson *et al.*, 1994) with those of the type strains of the 11 *Tenacibaculum* species and of other representative members of the family *Flavobacteriaceae* obtained from GenBank/EMBL. Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods (Fig. 1). The evolutionary distance matrix for the neighbour-joining method was generated according to Kimura's two-parameter model (Kimura, 1980). To evaluate phylogenetic trees, a bootstrap analysis with 1000 sample replications was performed with the SEQBOOT and CONSENSE programs in the PHYLIP 3.67 package (Felsenstein, 2007). The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). Results of morphological, physiological and biochemical tests are given in Table 1 and in the species description.

Fatty acid composition data for strain LL04 12.1.7$^T$ and type strains of other *Tenacibaculum* species are detailed in Table 2. The main difference between the novel isolate and the other *Tenacibaculum* type strains was the high content of unsaturated fatty acids (13.9 %) in strain LL04 12.1.7$^T$. The cellular fatty acid profile of strain LL04 12.1.7$^T$ was dominated by iso-$C_{15:0}$ (23.1 %), iso-$C_{15:0}$ 3-OH (10.6 %), iso-$C_{16:0}$ 3-OH (8.4 %), $C_{15:1}\omega6c$ (12.2 %) and summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 11.0 %) (Table 2).

The DNA G+C content of strain LL04 12.1.7 $^T$ was 29.8 mol%, the lowest reported value within the genus *Tenacibaculum*. Comparison of the 16S rRNA gene sequence of strain LL04 12.1.7$^T$ with those of the type strains of the 11 *Tenacibaculum* species and other members of the family *Flavobacteriaceae* demonstrated that strain LL04 12.1.7$^T$ formed a robust cluster with the *Tenacibaculum* species. The phylogenetic tree based on 16S rRNA gene sequences is shown in Fig. 1. The closest relatives of strain LL04 12.1.7$^T$ were the type strains of *T. ovolyticum* and *T. aestuarii* (96.7 % sequence similarity), *T. lutimaris* and *T. mesophilum* (96.4 %), *T. gallaicum* (96.2 %), *T. amylolyticum* (96.0 %), *T. litoreum* (95.9 %), *T. discolor* (95.8 %), *T. skagerrakense* (95.7 %), *T. litopenaei* (95.0 %) and *T. maritimum* (94.8 %). All these values are lower than the theoretical threshold (97 %) for the delineation of bacterial species based on 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). According to the phenotypic and genetic data obtained in this study, it is concluded that strain LL04 12.1.7$^T$ represents a novel species within the genus *Tenacibaculum*, for which the name *Tenacibaculum soleae* sp. nov. is proposed.

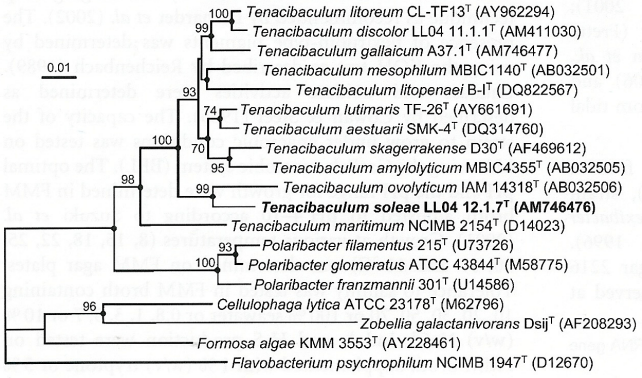


**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain LL04 12.1.7$^T$, other members of the genus *Tenacibaculum* and related genera of the family *Flavobacteriaceae*. Bootstrap values >50 % (based on 1000 replications) are shown at branching points. Solid circles indicate that the corresponding nodes are also recovered in the maximum-parsimony tree. Bar, 0.01 substitutions per site.

**Table 1.** Differential phenotypic characteristics of strain LL04 12.1.7$^T$ and the type strains of other *Tenacibaculum* species

Strains: 1, *T. soleae* sp. nov. LL04 12.1.7$^T$; 2, *T. gallaicum* DSM 18841$^T$; 3, *T. discolor* DSM 18842$^T$; 4, *T. mesophilum* MBIC1140$^T$; 5, *T. lutimaris* KCTC 12302$^T$; 6, *T. skagerrakense* ATCC BAA-458$^T$; 7, *T. amylolyticum* MBIC4355$^T$; 8, *T. ovolyticum* IAM 14318$^T$; 9, *T. maritimum* NCIMB 2154$^T$; 10, *T. litoreum* JCM 13039$^T$; 11, *T. aestuarii* KCTC 12569$^T$; 12, *T. litopenaei* BCRC 17590$^T$. Data from Wakabayashi *et al.* (1986), Hansen *et al.* (1992), Suzuki *et al.* (2001), Bernardet *et al.* (2002), Frette *et al.* (2004), Yoon *et al.* (2005), Choi *et al.* (2006), Jung *et al.* (2006), Sheu *et al.* (2007), Piñeiro-Vidal *et al.* (2008) and this study. +, Positive; −, negative; W, weakly positive; ND, no data available; NG, no growth.

| Character | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Colour | Yellow | Bright yellow | Bright yellow | Yellow | Pale yellow | Bright yellow | Yellow | Pale yellow | Pale yellow | Pale yellow | Pale yellow | Yellow |
| Cell size (μm) | 2–25 × 0.5 | 2–30 × 0.5 | 2–30 × 0.5 | 1.5–10 × 0.5 | 2–10 × 0.5 | 2–15 × 0.5 | 2–5 × 0.4 | 1.5–10 × 0.5 | 2–30 × 0.5 | 2–35 × 0.3–0.5 | 2–3.5 × 0.3 | 2–10 × 0.3–0.5 |
| Spherical cells in stationary phase | Frequent | Rare | Rare | Very rare | Very rare | Frequent | Very rare | ND | Frequent | Very rare | Very rare | Very rare |
| Gliding motility | + | + | + | + | + | − | + | + | + | + | + | + |
| Growth with: | | | | | | | | | | | | |
| NaCl (%) | NG | NG | NG | 1–7 | <8 (2–3) | NG | 3 | NG | NG | 3–5 | 2–3 | 2–10 |
| Seawater (%) | 55–100 | 30–100 | 30–100 | 10–100 | 25–175 | 25–150 | 50–100 | 70–100 | 30–100 | 25–250 | ND | ND |
| Temperature range (°C) | 14–30 | 14–38 | 14–38 | 15–40 | 10–39 | 10–40 | 20–35 | 4–25 | 15–34 | 5–40 | 9–41 | ND |
| Optimal growth temperature (°C) | 22–25 | 25–30 | 25–30 | 28–35 | 30–37 | 25–37 | 27–30 | ND | 25–30 | 35–40 | 30–37 | 28–37 |
| pH range | 6.0–8.0 | 6.0–8.0 | 6.0–8.0 | 5.3–9 | 7–8 | 6–9 | 5.3–8.3 | 5.9–8.6 | 5.9–8.6 | 6.0–10.0 | 5.5–8.5 | 7.0–8.0 |
| Degradation of: | | | | | | | | | | | | |
| Gelatin | + | + | + | − | + | + | − | − | + | − | + | + |
| Starch | − | − | − | + | − | + | + | + | + | + | − | + |
| Tween 80 | − | − | − | − | − | − | + | + | + | + | + | − |
| Carbon source utilization | | | | | | | | | | | | |
| (+)-D-Galactose | − | − | − | ND | ND | − | ND | ND | − | − | ND | ND |
| (+)-D-Glucose | − | − | − | ND | − | + | ND | ND | − | − | − | + |
| L-Proline | − | + | + | + | − | + | + | − | − | + | − | + |
| L-Glutamate | − | + | + | + | − | + | + | − | W | − | − | + |
| L-Tyrosine | − | − | − | ND | ND | ND | ND | ND | − | ND | ND | ND |
| API ZYM results | | | | | | | | | | | | |
| Esterase lipase (C8) | + | + | + | ND | ND | ND | ND | ND | + | − | + | + |
| Trypsin | − | + | + | ND | ND | ND | ND | ND | + | + | − | − |
| α-Chymotrypsin | − | + | + | ND | ND | ND | ND | ND | + | + | + | − |

**Table 2.** Cellular fatty acid compositions (%) of strain LL04 12.1.7$^T$ and the type strains of other *Tenacibaculum* species

Strains: 1, *T. soleae* sp. nov. LL04 12.1.7$^T$; 2, *T. mesophilum* MBIC1140$^T$; 3, *T. lutimaris* KCTC 12302$^T$; 4, *T. skagerrakense* ATCC BAA-458$^T$; 5, *T. maritimum* NCIMB 2154$^T$; 6, *T. litoreum* JCM 13039$^T$; 7, *T. aestuarii* KCTC 12569$^T$; 8, *T. litopenaei* BCRC 17590$^T$. Data from Yoon *et al.* (2005), Jung *et al.* (2006), Choi *et al.* (2006), Sheu *et al.* (2007) and this study. –, Not detected; tr, trace (<1 %); ECL, equivalent chain-length. No data were available for *T. ovolyticum*, *T. gallaicum* or *T. discolor*. Fatty acids amounting to less than 1 % of the total fatty acids in all strains tested are not listed.

| Fatty acid | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| **Straight chain** | | | | | | | | |
| $C_{15:0}$ | 4.8 | 3.6 | 8.9 | 4.9 | 2.9 | 2.7 | 6.1 | – |
| $C_{16:0}$ | tr | tr | tr | tr | tr | tr | tr | 1.8 |
| **Branched chain** | | | | | | | | |
| iso-$C_{13:0}$ | tr | tr | tr | tr | 1.8 | 1.4 | 1.3 | tr |
| iso-$C_{14:0}$ | 2.4 | tr | 1.7 | tr | tr | 2.2 | 2.2 | tr |
| iso-$C_{15:0}$ | 23.1 | 13.2 | 17.2 | 9.5 | 16.8 | 18.8 | 18.9 | 22.0 |
| anteiso-$C_{15:0}$ | tr | 1.1 | tr | – | tr | 1.8 | 2.0 | tr |
| iso-$C_{15:1}$ | 5.7 | 7.1 | 5.3 | 8.2 | 7.6 | 8.2 | 8.7 | 8.7 |
| iso-$C_{16:0}$ | 1.7 | 1.7 | 3.8 | 1.3 | tr | 2.3 | 2.3 | 1.8 |
| iso-$C_{16:1}$ | 2.4 | tr | 1.7 | 1.7 | – | 1.3 | tr | 1.6 |
| iso-$C_{17:1}\omega 9c$ | – | tr | tr | tr | – | 1.6 | 1.3 | 1.6 |
| **Unsaturated** | | | | | | | | |
| $C_{15:1}\omega 6c$ | 12.2 | 1.6 | 4.2 | – | 2.2 | 1.7 | 3.0 | 1.6 |
| $C_{17:1}\omega 6c$ | 1.7 | tr | 1.5 | 1.2 | tr | tr | 1.6 | 1.9 |
| $C_{18:3}\omega 6c$ | – | – | – | – | – | 1.5 | – | – |
| **Hydroxylated** | | | | | | | | |
| iso-$C_{15:0}$ 3-OH | 10.6 | 8.0 | 4.6 | 7.8 | 19.8 | 6.6 | 6.1 | 4.6 |
| $C_{15:0}$ 2-OH | tr | 1.1 | 1.2 | 2.5 | 1.1 | tr | tr | tr |
| $C_{15:0}$ 3-OH | 3.2 | 2.9 | 3.4 | 8.6 | 3.8 | – | 4.2 | 2.7 |
| iso-$C_{16:0}$ 3-OH | 8.4 | 9.0 | 12.8 | 12.2 | 5.0 | 6.8 | 12.3 | 3.4 |
| $C_{16:0}$ 3-OH | 2.2 | 3.2 | 1.3 | 2.1 | 1.5 | 1.6 | 1.0 | 5.4 |
| iso-$C_{17:0}$ 3-OH | 2.9 | 14.9 | 8.4 | 11.7 | 13.7 | 13.6 | 9.6 | 12.7 |
| $C_{17:0}$ 3-OH | – | tr | tr | 2.5 | – | tr | tr | 1.0 |
| **Unknown fatty acids** | | | | | | | | |
| ECL 13.565 | tr | tr | – | – | – | – | – | 1.9 |
| ECL 16.582 | tr | 1.0 | tr | tr | 1.0 | 1.0 | 1.0 | tr |
| **Summed features*** | | | | | | | | |
| Summed feature 3 | 11.0 | 24.4 | 18.1 | 22.5 | 17.9 | 19.6 | 11.9 | 21.3 |
| Summed feature 4 | – | – | – | – | – | 1.3 | – | – |

*Summed feature are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH. Summed feature 4 comprises iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ H.

### Description of *Tenacibaculum soleae* sp. nov.

*Tenacibaculum soleae* [so.le'ae. L. gen. n. *soleae* of a sole, in reference to the source of the isolate, a cultured sole (*Solea senegalensis* Kaup)].

Cells are Gram-negative rods, 0.5 μm in diameter and 2–25 μm in length, motile by gliding. Spherical cells are observed in ageing cultures. Colonies on FMM agar and MA 2216 (Difco) are flat and yellow with uneven edges and do not adhere to the agar. The yellow pigment does not belong to the flexirubin type. Strictly aerobic. Growth occurs in media containing 50–100 % seawater, but not in media supplemented with NaCl only. Growth occurs at 14–30 °C (optimum 22–25 °C) and pH 6.0–8.0. Catalase and cytochrome oxidase activities are present. Gelatin and casein are hydrolysed, but Tween 80 and starch are not. The Voges–Proskauer test is negative. No acid is produced from carbohydrates. $H_2S$ and indole are not produced. L-Proline, L-glutamate, sucrose, (–)-D-ribose, (+)-D-galactose, (+)-D-glucose and L-tyrosine are not utilized. In the API ZYM system, alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase and cystine arylamidase activities are present, but trypsin, α-chymotrypsin and all enzymes related to the metabolism of carbohydrates are absent.

The type strain is LL04 12.1.7$^T$ (=CECT 7292$^T$ =NCIMB 14368$^T$), isolated from a diseased sole (*Solea senegalensis*) reared in Galicia (north-western Spain).

## Acknowledgements

This investigation was supported by grant PGIDIT 04 RMA 003E from the Xunta de Galicia, Spain. The authors acknowledge Stolt Sea Farm for the collaboration provided for the study, Dr J. P. Euzéby (Laboratoire de Bactériologie, École Nationale Vétérinaire, Toulouse, France) for his help with species names and Dr David Arahal (Colección Española de Cultivos Tipo, CECT, Burjassot, Valencia, Spain) for constructive suggestions.

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**Artículo nº5**. Piñeiro-Vidal, M. Pazos, F., Santos, Y. (2008). Fatty acid as a chemotaxonomic tool for taxonomic and epidemiological characterization of four fish pathogenic *Tenacibaculum* species. *Letters in Applied Microbiology* **46**, 548-554.

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

# Fatty acid analysis as a chemotaxonomic tool for taxonomic and epidemiological characterization of four fish pathogenic *Tenacibaculum* species

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**Keywords**
fatty acid, flexibacteriosis, identification, multivariate analysis, *Tenacibaculum discolor*, *Tenacibaculum gallaicum*, *Tenacibaculum maritimum*, *Tenacibaculum ovolyticum*.

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2007/1362: received 29 August 2007, revised 16 January 2008 and accepted 21 January 2008

doi:10.1111/j.1472-765X.2008.02348.x

## Abstract

**Aims:** In this work, fatty acid content and profiles were analysed in order to differentiate the species *Tenacibaculum maritimum*, *Tenacibaculum gallaicum*, *Tenacibaculum discolor* and *Tenacibaculum ovolyticum* that are pathogenic for cultured marine fish and to assess the potential of fatty acid profiles as a tool for epizootiological typing.
**Methods and Results:** The fatty acid methylesters (FAMEs) were extracted from cells grown on marine agar for 48 h at 25°C and were prepared and analysed according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System. The cellular fatty acid profiles of *Tenacibaculum* strains tested were characterized by the presence of large amounts of branched (36·1–40·2%) and hydroxylated (29·6–31·7%) fatty acids. The FAME products from the four species significantly ($P < 0{\cdot}05$) differed in the content of iso-$C_{15:0}$3-OH, iso-$C_{16:0}$3-OH, iso-$C_{15:1}$G, summed feature 3 (a component that contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{16:0}$, $C_{17:1}\omega6c$, $C_{15:0}$3-OH, iso-$C_{17:0}$3-OH.
**Conclusions:** Results of present study demonstrated the existence of differences in the fatty acids content between the *T. maritimum* isolates from different marine fish/geographical origin and between strains of *T. maritimum*, *T. discolor*, *T. gallaicum* and *T. ovolyticum*.
**Significance and Impact of the Study:** Profiling of fatty acids may be a useful tool to distinguish *T. maritimum* from other *Tenacibaculum* species pathogenic for fish as well as for epizootiological differentiation of *T. maritimum* isolates.

## Introduction

Marine flexibacteriosis affects salmonid and nonsalmonid fish worldwide and is considered a limiting factor for the culture of many economically important fish species (Wakabayashi *et al.* 1986; Bernardet *et al.* 1990; Pazos *et al.* 1993; Chen *et al.* 1995; Handlinger *et al.* 1997; Ostland *et al.* 1999; Cepeda and Santos 2002; Avendaño-Herrera *et al.* 2004), causing serious mortalities in farms. Although the filamentous organism *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) is the best known causative agent of flexibacteriosis in marine fish, we have recently characterized other filamentous gliding bacteria associated with disease in juveniles and adult stages of turbot and sole as well as from seawater in fish-holding tanks. These bacteria showed similar phenotypic characteristics and virulence degree that *T. maritimum* (Piñeiro-Vidal *et al.* 2007). Genetic analyses demonstrated that these filamentous bacteria belong to the genus *Tenacibaculum* but are different from the other validly published species (Piñeiro-Vidal *et al.* 2008). Bernardet *et al.* (2002) have proposed minimal standards for describing new taxa on the family *Flavobacteriaceae*. In addition to phenotypic characteristics, genomic analysis, relationship to the host and whole-cell protein analysis, these authors indicated that the presence or amount of some fatty acids could be of value to differentiate a new taxon from existing taxa of the genus. Chemotaxonomic analysis has also been

successfully applied in taxonomy study to many environmentally, clinically or biotechnologically relevant species (Komagata and Suzuki 1987; Welch 1991; Vandamme *et al.* 1996; Steele *et al.* 1997; Dijkshoorn and Towner 2001; Inglis *et al.* 2003; Shoemaker *et al.* 2005). However, a limited number of studies have attempted to use fatty acid profiling as a tool for epidemiologic typing (Welch 1991; Birnbaum *et al.* 1994; Steele *et al.* 1997; Haussler *et al.* 1998; Inglis *et al.* 2003), including other fish pathogens (Shoemaker *et al.* 2005). The main objective of this study was to assess the potential of fatty acid profiles in the taxonomic and epizootiological differentiation of *T. maritimum* strains from other fish pathogenic bacteria of the genus *Tenacibaculum*.

## Materials and methods

### Bacterial strains and growth conditions

Strains of *T. maritimum* (32 strains), *Tenacibaculum ovolyticum* (one strain), *Tenacibaculum discolor* (seven strains) and *Tenacibaculum gallaicum* (seven strains) isolated from marine fish culture systems in different geographical areas (Table 1) were used in the present study. The bacteria were grown on marine agar 2216 (MA; Difco, Le Pont de Claix, France) at 25°C for 48 h. Stock cultures of the strains were maintained at −70°C in Microbank™ tubes (Prolab Diagnostics, ON, Canada). The taxonomic position of all the strains were confirmed by using conventional morphological, physiological and biochemical tests, APIZYM (bioMérieux, Marcy l'Etoile, France) and serological methods as previously described (Piñeiro-Vidal *et al.* 2007, 2008), prior to use in generating whole-cell fatty acid profiles.

### Analysis of fatty acid methylesters (FAMEs)

For fatty acid analysis, the strains were grown on MA plates for 48 h at 25°C. One representative strain of each species (strain LL01 8.3.8 of *T. maritimum*, strain LL04.11.1.1 of *T. discolor*, A37.1 of *T. gallaicum* and DSM 18103 of *T. ovolyticum*) was analysed in duplicate in order to evaluate the fatty acid profile variability in independent cultures of the same bacterium. Cell harvesting, saponification of lipids, methylation of fatty acids, extraction of fatty acid methyl esters, washing of extracts and gas chromatography analysis were performed according to the standardized procedures for the Microbial Identification System (MIDI; Microbial ID Inc., Newark, DE, USA) (Sasser 1990). Data from *T. maritimum*, *T. discolor*, *T. gallaicum* and *T. ovolyticum* isolates were exported to the XLSTAT software package and dendrograms were generated on the basis of the unweigthed average pair group (Sneath and Sokal 1973) after Pearson's product–moment correlation analysis. The existence of significant differences in the amount of individual fatty acids produced by strains belonging to the three *Tenacibaculum* species was determined by ANOVA using SPSS version 14.0 statistic software package. In order to evaluate the contribution of the major and minor fatty acids in the differentiation of *Tenacibaculum* strains, two-dimensional plot of principal components (PC) was performed using XLSTAT software package.

## Results

The fatty acid compositions of *T. maritimum*, *T. discolor*, *T. gallaicum* and *T. ovolyticum* strains are detailed in Table 2. The fatty acids that were found in trace quantities (<1·0%) in all the strains are not shown. As identical culture conditions were used for all strains studied, the resulting fatty acid profiles showed only very little quantitative variation in independent cultures of the same organism. The cellular fatty acid profiles of *Tenacibaculum* strains tested were characterized by the presence of large amounts of branched (36·1–40·2%) and hydroxylated (29·6–31·7%) fatty acids (Table 2). *Tenacibaculum maritimum* contained 32 different fatty acids. The most abundant were iso-$C_{15:0}$, followed by iso-$C_{15:0}$3-OH, summed feature 3 (a component that contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{15:1}$G, iso-$C_{17:0}$3-OH, iso-$C_{16:0}$3-OH. *Tenacibaculum discolor* possessed 37 types of fatty acids with a predominance of iso-$C_{15:0}$, followed by summed feature 3, iso-$C_{16:0}$3-OH, iso-$C_{17:0}$3-OH, iso-$C_{15:1}$G and iso-$C_{15:0}$3-OH. For *T. gallaicum* isolates 34 different fatty acids were detected. The major components were iso-$C_{15:0}$, summed feature 3, iso-$C_{17:0}$3-OH, iso-$C_{16:0}$3-OH, iso-$C_{15:1}$G, iso-$C_{15:0}$3-OH. *Tenacibaculum ovolyticum* contained 36 different fatty acids. The most abundant were iso-$C_{15:0}$3-OH, followed by iso-$C_{15:0}$, $C_{15:1}\omega6c$, iso-$C_{14:0}$, iso-$C_{15:1}$G, iso-$C_{13:0}$, $C_{16:0}$3-OH, $C_{15:0}$, $C_{15:0}$3-OH. FAMEs from the four species significantly ($P < 0{\cdot}05$) differed in the content of iso-$C_{15:0}$3-OH, iso-$C_{16:0}$3-OH, iso-$C_{15:1}$ G, summed feature 3, iso-$C_{16:0}$, $C_{17:1}\omega6c$, $C_{15:0}$3-OH and iso-$C_{17:0}$3-OH (Table 2). As the genus *Tenacibaculum* is not included in the MIDI databases of clinical and environmental bacteria, most of the strains tested were not identified or were misidentified as *Zobellia uliginosa* or *Flavobacterium johnsoniae* (*Cytophaga johnsonae*). The result of principal components analysis is shown in Fig. 1. This analysis allowed us to evaluate the contribution of each fatty acid in the differentiation of strains of *T. maritimum*, *T. discolor*, *T. gallaicum* and *T. ovolyticum* analysed and to determine the taxonomic relationships between these strains. The projection of the samples in the plane defined by the axes 1 (representing

**Table 1** *Tenacibaculum* strains used to generate fatty acid profiles

| Strain | Identification | Source | Year of isolation |
|---|---|---|---|
| NCIMB2153 | *T. maritimum* | Blackhead seabream (*Acanthopagrus schlegeli,* Blecker), Japan | 1976 |
| Lyl-1 | *T. maritimum* | Blackhead seabream (*A. schlegeli,* Blecker), Japan | 1985 |
| NCIMB2154 | *T. maritimum* | Japanese seabream (*Pagrus major,* Temminck & Schlegel), Japan | 1977 |
| FPC394 | *T. maritimum* | Japanese seabream (*P. major,* Temminck & Schlegel), Japan | 1982 |
| NCIMB2158 | *T. maritimum* | Sole (*Solea solea,* L.), UK | 1981 |
| JIP21/91 (2) | *T. maritimum* | Sea bass (*Dicentrarchus labrax*, L.), France | 1991 |
| JIP32/91 | *T. maritimum* | Sea bass (*D. labrax*, L.), France | 1991 |
| GBF 8601 | *T. maritimum* | Bastard halibut (*Paralychthys olivaceous*, Temminck & Schlegel), Japan | 1986 |
| DBA 4 a | *T. maritimum* | Japanese amberjack (*Seriola quinqueradiata,* Temminck & Schlegel), Japan | 1986 |
| FES342/01 | *T. maritimum* | Giltheah seabream (*Sparus aurata,* L.), Spain | 2001 |
| DOB 102 | *T. maritimum* | Giltheah seabream (*S. aurata,* L.), Spain | 2002 |
| UCD6F | *T. maritimum* | Californian anchoveta (*Engraulis mordaz, Girard*), USA | 1994 |
| UCD WSB-1b | *T. maritimum* | White sea bass (*Atractoscion mobilis*, Ayres), USA | |
| LL01 7.3.1 | *T. maritimum* | Sole (*Solea senegalensis,*Kaup), Spain | 2001 |
| LL01 8.3.8 | *T. maritimum* | Sole (*S. senegalensis*, Kaup), Spain | 2001 |
| LL01 10.5.3 | *T. maritimum* | Sole (*S. senegalensis*, Kaup), Spain | 2002 |
| LL03 6.2.1 | *T. maritimum* | Sole (*S. senegalensis*, Kaup), Spain | 2003 |
| LL03 7.5.2 | *T. maritimum* | Sole (*S. senegalensis*, Kaup), Spain | 2003 |
| LC07 10.3.1 | *T. maritimum* | Sole (*S. senegalensis*, Kaup), Spain | 2007 |
| LC07 10.1.2 | *T. maritimum* | Sole (*S. solea*, L.), Spain | 2007 |
| JIP 10/97 | *T. maritimum* | Turbot (*Psetta maxima*, L.), France | 1997 |
| JIP 31/99 | *T. maritimum* | Turbot (*P. maxima*, L.), France | 1999 |
| RA107.1 | *T. maritimum* | Turbot (*P. maxima*, L.), France | 2007 |
| Tm Chile | *T. maritimum* | Turbot (*P. maxima*, L.), Chile | 1998 |
| C02 4.1.3 | *T. maritimum* | Turbot (*P. maxima*, L.), Spain | 2002 |
| C03 6.1.3 | *T. maritimum* | Turbot (*P. maxima*, L.), Spain | 2003 |
| C04 3.2.1 | *T. maritimum* | Turbot (*P. maxima*, L.), Spain | 2004 |
| LPV1.7 | *T. maritimum* | Turbot (*P. maxima*, L.) | 1995 |
| C02 8.1.1 | *T. maritimum* | Turbot (*P. maxima*, L.) | 2002 |
| C02 11.2.1 | *T. maritimum* | Turbot (*P. maxima*, L.) | 2002 |
| C03 11.2.1 | *T. maritimum* | Turbot (*P. maxima*, L.) | 2003 |
| LS95 2.2.1 | *T. maritimum* | Sole (*S. solea* L.) | 1995 |
| LL04 11.1.1 | *Tenacibaculum discolor* | Sole (*S. senegalensis*, Kaup) | 2004 |
| LC06 11.5.1 | *T. discolor* | Sole (*S. senegalensis* Kaup) | 2006 |
| LC07 3.2.2 | *T. discolor* | Sole (*S. senegalensis* Kaup) | 2007 |
| ITd07.2 | *T. discolor* | Turbot (*P. maxima* L.) | 2007 |
| ITd07.4 | *T. discolor* | Turbot (*P. maxima* L.) | 2007 |
| ITd07.5 | *T. discolor* | Turbot (*P. maxima* L.) | 2007 |
| A22.2 | *T. discolor* | Fish holding tanks | 2003 |
| R006.0.1 | *Tenacibaculum gallaicum* | Turbot (*P. maxima* L.) | 2000 |
| R006.0.3 | *T. gallaicum* | Turbot (*P. maxima* L.) | 2000 |
| CLT07.1 | *T. gallaicum* | Turbot (*P. maxima* L.) | 2007 |
| LC07 10.2.2 | *T. gallaicum* | Sole (*S. senegalensis*, Kaup) | 2007 |
| A37.1 | *T. gallaicum* | Seawater in fish-holding tanks | 2003 |
| A13.3 | *T. gallaicum* | Seawater in fish-holding tanks | 2003 |
| A35.1 | *T. gallaicum* | Seawater in fish-holding tanks | 2003 |
| DSM 18103 | *Tenacibaculum ovolyticum* | Halibut (*Hippoglossus hippoglossus*, L.) eggs, Norway | 1989 |

35·1% of the variance) and 2 (26·3% of the variance) revealed the existence of four groups. Group I, included strains of *T. maritimum*, characterized by the presence of iso-$C_{15:1}$ G and iso-$C_{15:0}$ 3-OH. Two subgroups (Ia and Ib) associated with the isolation source (host/geographical origin) of the strain were observed. The subgroup Ia included the majority of *T. maritimum* strains tested that were characterized by its high content of iso-$C_{15:1}$G (>10·5%). The subgroup Ib included all the strains isolated from *S. senegalensis*, three strains isolated from turbot in different geographical area (Tm Chile, RA107.1 and UCD WSB-1b) characterized by its high content

**Table 2** Major fatty acids of *Tenacibaculum* strains studied

| Fatty acid* | Mean % composition (Standard error)† | | | |
|---|---|---|---|---|
| | *T. maritimum* (*n* = 32) | *T.discolor* (*n* = 7) | *T. gallaicum* (*n* = 7) | *T. ovolyticum* (*n* = 1) |
| Straight chain | | | | |
| $C_{15:0}$ | 1·4 ± 0·3 | 3·0 ± 1·0 | 1·7 ± 0·5 | 5·6 |
| Branched chain | | | | |
| Iso-$C_{13:0}$ | 1·3 ± 0·0 | Tr‡ | Tr | 6·9 |
| Iso-$C_{14:0}$ | Tr | 1·1 ± 0·0 | Tr | 8·0 |
| Iso-$C_{15:0}$ | 20·8 ± 1·1 | 18·1 ± 0·9 | 21·2 ± 0·6 | 11·1 |
| anteiso-$C_{15:0}$ | 1·3 ± 0·0 | 1·1 ± 0·2 | 1·6 ± 0·2 | 3·5 |
| Iso-$C_{15:1}$ G | 12·1 ± 0·4 | 8·1 ± 0·4 | 8·9 ± 0·3 | 7·9 |
| Iso-$C_{16:0}$ | Tr | 3·3 ± 0·1 | 2·1 ± 0·4 | 0·6 |
| Iso-$C_{16:1}$ H | 1·9 ± 0·1 | 2·7 ± 0·2 | 1·9 ± 0·1 | ND§ |
| Iso-$C_{17:1}$ $\omega$9c | 1·2 ± 0·1 | 1·1 ± 0·0 | 1·1 ± 0·1 | ND |
| Unsaturated | | | | |
| $C_{15:1}$ $\omega$6c | 3·9 ± 0·2 | 3·2 ± 0·1 | 2·8 ± 0·4 | 8·2 |
| $C_{17:1}$ $\omega$6c | Tr | 1·7 ± 0·0 | 1·6 ± 0·3 | 1·3 |
| Hydroxylated | | | | |
| $C_{15:0}$ 3OH | 1·4 ± 0·2 | 2·3 ± 0·0 | 2·5 ± 0·2 | 5·2 |
| Iso-$C_{15:0}$ 3OH | 14·8 ± 0·4 | 5·2 ± 0·2 | 6·7 ± 0·2 | 14·8 |
| $C_{16:0}$ 3OH | 1·2 ± 0·1 | 1·4 ± 0·1 | 1·9 ± 0·1 | 6·2 |
| Iso-$C_{16:0}$ 3OH | 5·9 ± 0·3 | 11·9 ± 0·2 | 10·0 ± 0·3 | 2·1 |
| Iso-$C_{17:0}$ 3OH | 8·5 ± 0·3 | 9·1 ± 0·4 | 10·5 ± 0·2 | 1·4 |
| Unknown 16·582 | Tr | 1·0 ± 0·0 | 1·1 ± 0·0 | ND |
| Summed feature 3§ | 13·9 ± 0·2 | 17·9 ± 0·3 | 16·4 ± 0·6 | 4·8 |

*The fatty acids that had mean values of <1% in all strains studied are not listed.
†Mean and standard error were calculated regardless of the fact that a particular fatty acid was not produced by all strains analysed.
‡Tr, traces, values lower than 1·0%.
§ND, not detected.
¶Summed feature 3 contains $C_{16:1}$ $\omega$7c and/or iso-$C_{15:0}$ 2OH that cannot be separated by gas chromatography using the microbial identification system.

of iso-$C_{15:0}$3OH (>14·0) and one strain isolated from bastard halibut in Japan (GBF8601). Group II, included all *T. discolor* strains, strongly associated with the presence of iso-$C_{16:0}$, iso- $C_{16:0}$ 3-OH and the summed feature 3. Group III, included the *T. gallaicum* isolates, characterized by the presence of iso-$C_{17:0}$3-OH was closely related to group II. Group IV, included the type strain of *T. ovolyticum*, characterized by the presence of $C_{15:0}$, iso-$C_{13:0}$, iso-$C_{14:0}$, anteiso-$C_{15:0}$, $C_{15:1}\omega$6c, $C_{15:0}$ 3-OH, $C_{16:0}$ 3-OH. Similar grouping was obtained with FAME-based similarity dendrogram constructed from Pearson's product–moment correlation coefficients (data not shown).

## Discussion

This study describes results from fatty acid analysis of strains of *T. maritimum*, *T. discolor*, *T. gallaicum* and *T. ovolyticum* isolated from different fish species from distinct geographical areas. In general, the fatty acid profile of *Tenacibaculum* strains tested in the present work was in accordance with those previously published for the genus *Tenacibaculum* (Bowman *et al.* 1998; Jung *et al.* 2006; Sheu *et al.* 2007) although there were some differences in the amounts of some fatty acids that can be attributed to the different culture conditions (medium, temperature and incubation period), strains and/or the analytical procedures used. The FAME profiles of *T. maritimum*, *T. discolor* and *T. gallaicum* strains did not significantly differed except for a few fatty acids (iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH, iso-$C_{15:1}$ G, summed feature 3, iso-$C_{16:0}$ and $C_{17:1}$ $\omega$6c) (Table 2). However, the FAME profile of *T. ovolyticum* showed notable quantitative and qualitative differences with respect to those observed in the other fish pathogenic *Tenacibaculum* species analysed. Interestingly, it was observed that strains of *T. maritimum* isolated from different fish species and/or geographical areas differed significantly in the amounts of some fatty acids (iso-$C_{15:0}$ 3-OH and iso- $C_{15:1}$ G) (Table 2), suggesting that FAME profiling could be used to distinguish strains isolated from different sources.

As shown in the two-dimensional PC plots, *T. ovolyticum* strain was clearly separated from strains of the other

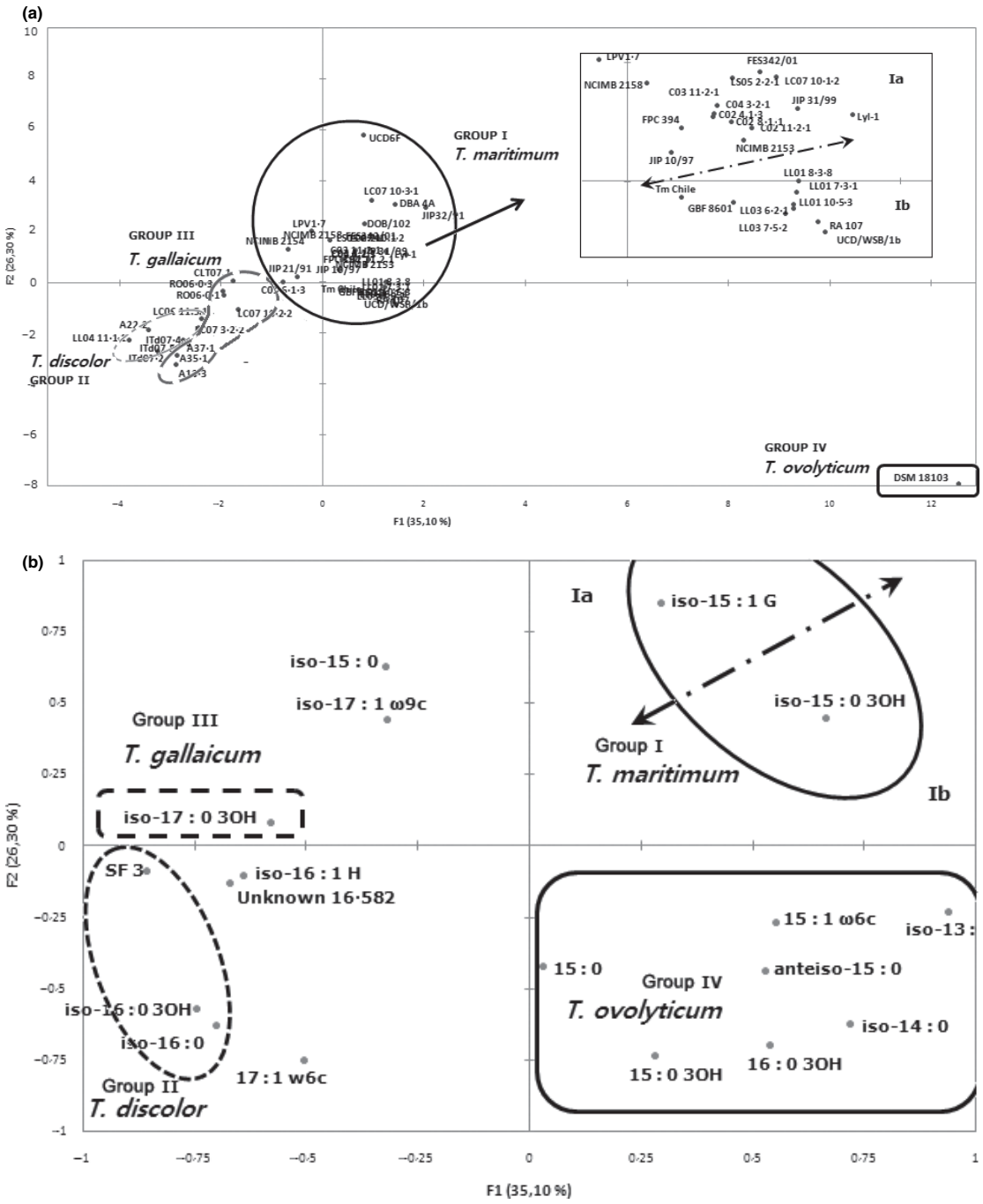


**Figure 1** Two-dimensional plot of principal components of fatty acid methylesters of *Tenacibaculum* strains tested. (a) Projection of the bacterial cultures in the plane defined by the two main axes of the principal component analysis. The inertia of the axes 1 and 2 are 35·1% and 26·3%, respectively. Group I, 32 strains of *Tenacibaculum maritimum*; Group II, seven strains of *Tenacibaculum discolor* isolated from sole; Group III, seven strains of *Tenacibaculum gallaicum*; Group IV, Type strain of *T. ovolyticum*. (b) Projection of 18 fatty acids detected in the plane defined by the axes 1 and 2. Fatty acids that are important for the establishment of groups are indicated.

*Tenacibaculum* species tested. Moreover, *T. maritimum* strains isolates were separated in two groups associated with the host/geographic origin. Fatty acids iso-$C_{15:1}$ G and iso-$C_{15:0}$ 3-OH had the most important contribution to the clustering of the strains of *T. maritimum* and may be used as chemotaxonomic markers for epidemiological studies. Further studies with a high number of strains of *T. maritimum* isolated from turbot, sole and other marine fish species in other geographical areas will help to clarify if the differences observed between the fish species are because of a fish specificity of the pathogen. In contrast, *T. discolor* and *T. gallaicum* strains were very closely related based on their fatty acid composition. These results are congruent with our preliminary phylogenetic study where *T. discolor* and *T. gallaicum* strains are not distinguished on the basis of the 16S rRNA gene sequences similarity (similarity levels of 98·4%) (Piñeiro-Vidal *et al.* 2008).

The overall results of the present study suggest that cellular fatty acid analysis could decisively facilitate distinction of *T. maritimum* from other bacteria of the genus *Tenacibaculum* pathogenic for marine fish and even differentiate strains from different isolation sources. In a recent study, Shoemaker *et al.* (2005) also concluded that fatty acid analysis will allow reliable identification of the freshwater fish pathogen *Flavobacterium columnare*.

## Acknowledgements

This investigation was supported by grants PGIDIT 04 RMA 003E and PGIDIT 06 RMA 024E from the Xunta de Galicia, Spain. The authors acknowledge Gonzalo Álvarez (Dpto. Genética, Universidad de Santiago de Compostela) for the collaboration provided with the SPSS software.

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# Capítulo IV B. Láminas complementarias

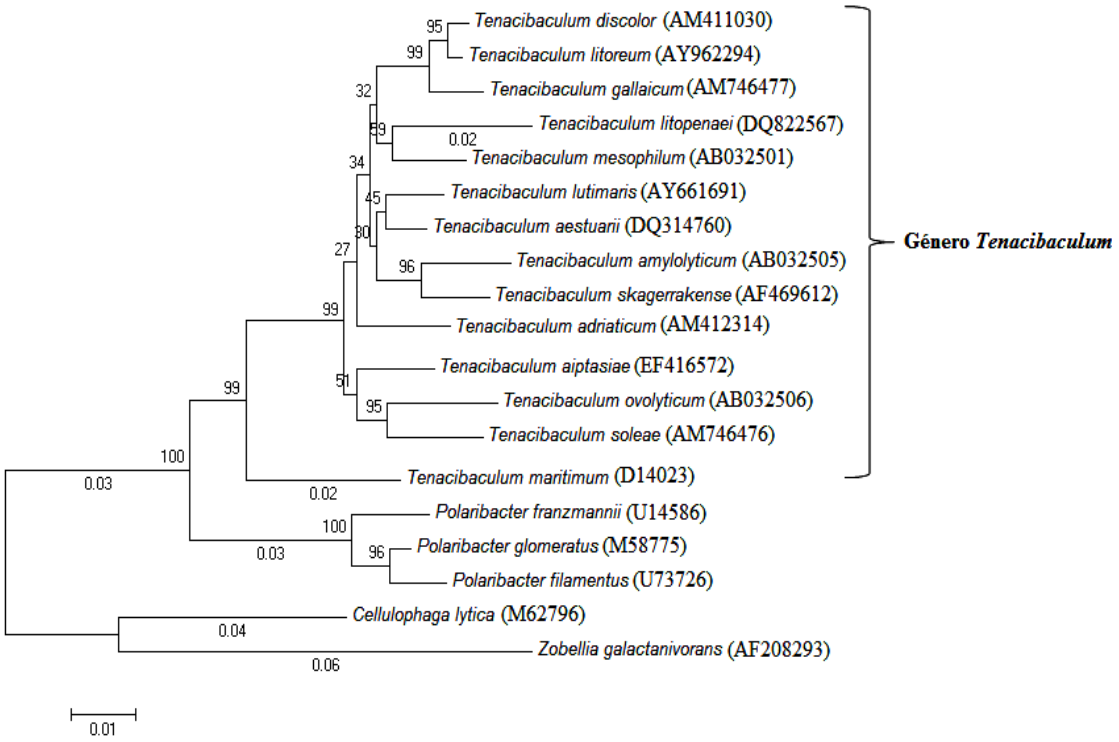


Lámina 5.- Árbol filogenético mostrando la relación entre las 14 cepas tipo representantivas de las especies del género *Tenacibaculum*, y otras especies relacionadas pertenecientes a la familia *Flavobacteriaceae*. El análisis se basó en las secuencias del ARNr 16S usando el método Neighbour-Joining (Saitou and Nei, 1987). Para el análisis se realizaron 1000 replicaciones indicando los números el porcentaje de aparición del nexo. La barra inferior indica sustituciones nucleotídicas por posición.

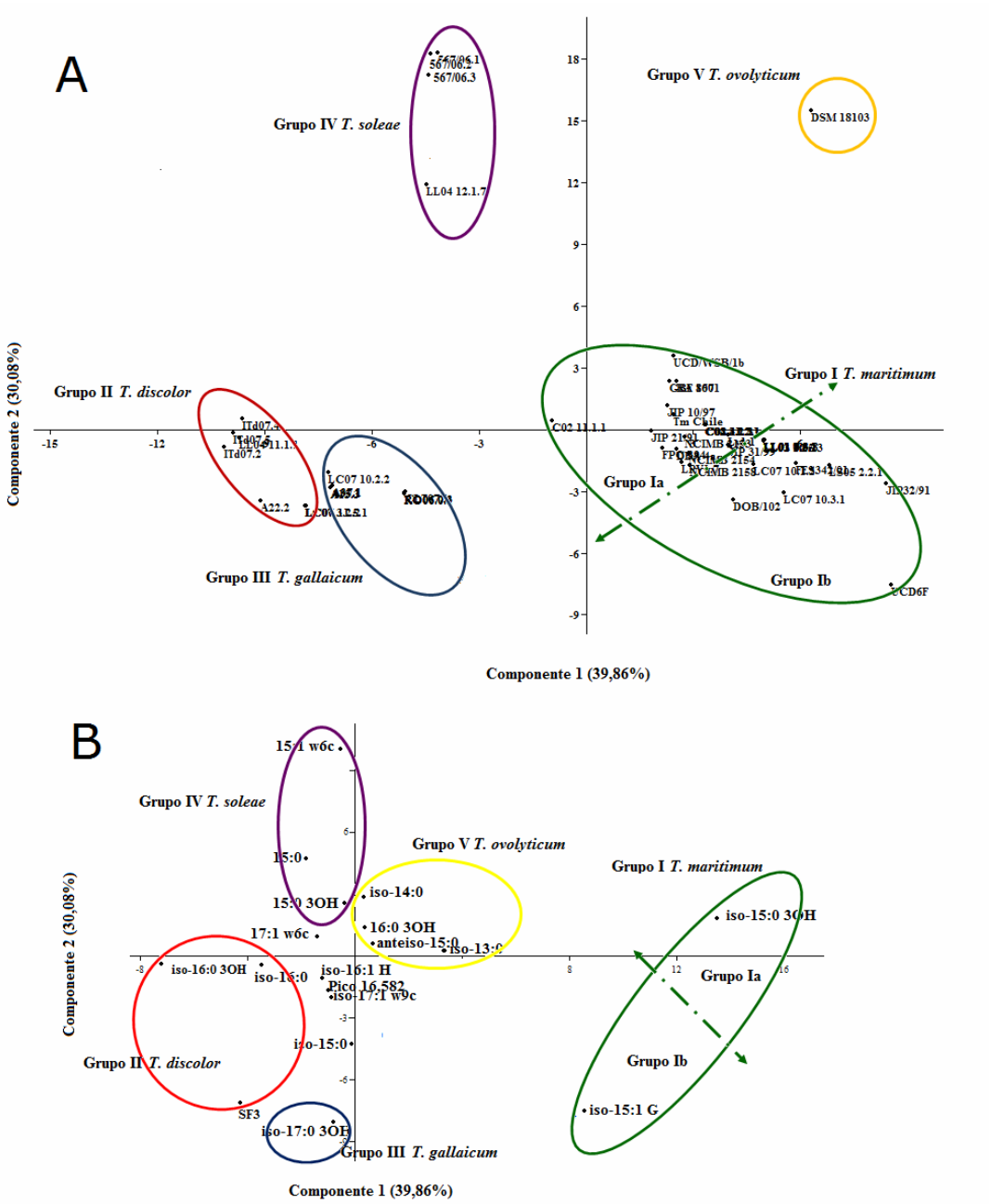


Lámina 6.- Análisis de componentes principales (PCA) de los perfiles de ácidos grasos de las especies patógenas de peces pertenecientes al género *Tenacibaculum* (A) y proyección de los ácidos grasos en el plano XY (B). Los porcentajes en los ejes X e Y indican la representatividad de las dos variables sobre el total de las mismas.

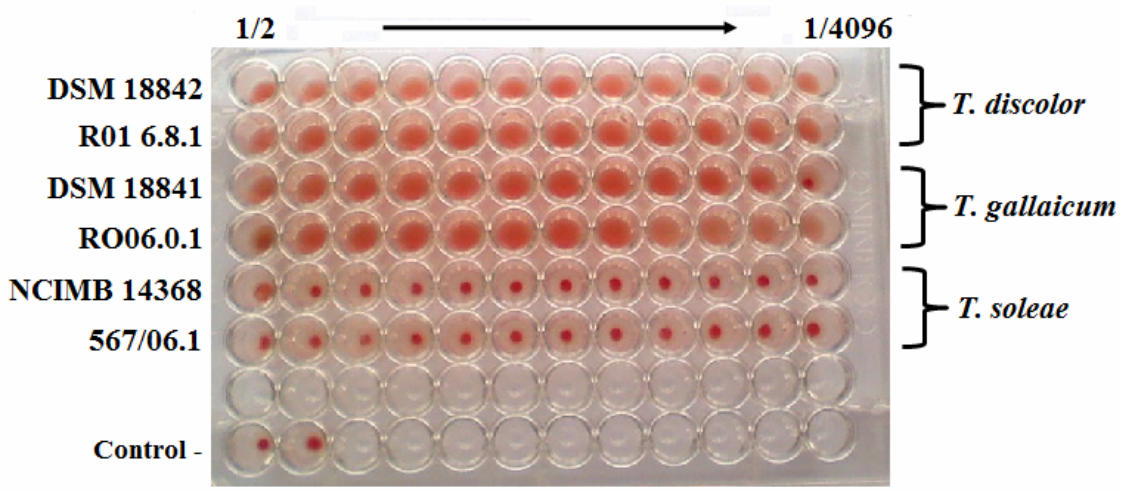


Lámina 7.- Producción de hemolisinas a las 48 horas por parte de las bacterias pertenecientes al género *Tenacibaculum* en estudio.

# Capítulo V Caracterización inmunológica de *Tenacibaculum* spp. patógenas para lenguado y rodaballo

# V A. Publicaciones

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**Artículo nº 6**. Piñeiro-Vidal, M., Riaza, A. & Santos, Y. (2006) Serological typing of *Tenacibaculum* sp. isolated from diseased turbot and sole cultured in Spain. In: **Proceedings of Aquamedit 2006**. (M. Chatziefstathiou, Eds.), pp; 1-10. Pireaus, Greece.

3rd International Congress on Aquaculture, Fisheries Technology and Environmental Management
3 – 4 November 2006, Athens, Greece, EU

# SEROLOGICAL TYPING OF *Tenacibaculum* sp. ISOLATED FROM DISEASED TURBOT AND SOLE CULTURED IN SPAIN

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## Abstract

This work reports on the serological characterization of *Tenacibaculum* sp. isolated from diseased turbot and sole and from seawater in fish-holding tanks in the Northwest of Spain. Reference strains from *T. maritimum* and *T. litoreum* were included for comparison. The serological analysis demonstrated that 6 of 17 (35.3%) *Tenacibaculum* sp strains were typeable being distributed into 4 serological O-groups.

There was not cross reaction between groups. Neither the *Tenacibaculum* sp strains isolated from fish or seawater nor the reference strain of *T.litoreum* JCM13039 reacted with the anti-*Tenacibaculum maritimum* NCIMB2153 serum. Moreover, strains of *Tenacibaculum maritimum* and *Tenacibaculum litoreum* did not react with sera anti-A37.1, anti- R01.6.8.1 and anti-LL0411.1.1, supporting that strains of filamentous bacteria analysed in the present study do not belong to the species *T. maritimum* or *T. litoreum*.

**Key words:** *Tenacibaculum* sp., serotypes, immunoblot, LPS, membrane proteins, fish pathogens

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3 – 4 November 2006, Athens, Greece, EU

## Introduction

Marine flexibacteriosis is widely distributed in cultured and wild fish in Europe, Japan and USA and is considered a potential limiting factor for the culture of economically important marine fish species such as turbot (*Scophthalmus maximus*), sole (*Solea solea* and *Solea senegalensis*), sea bass (*Dicentrarchus labrax*), salmon (*Salmo salar* and *Oncorhynchus kisutch*), red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli)* and flounder (*Paralichthys olivaceus*) (Wakabayashi *et al*., 1986; Bernardet *et al*., 1990; Bernardet, 1997; Pazos *et al.* 1993; Santos *et al*., 1999; Cepeda and Santos, 2002), causing serious mortalities in farms. Infected fish have eroded and haemorrhagic mouth, ulcerative skin lesions, frayed fins, and tail rot. A systemic disease can be also established involving different internal organs. Infected tissues can appear pale-yellow due to the presence of large number of bacteria. (Bernardet, 1997; Santos *et al*., 1999).

In Galicia (Northwest of Spain) flexibacteriosis is a significant disease problem for the culture of turbot and sole. *Tenacibaculum maritimum* of serotypes O1, O2, and O3 and, in a lower extent, the serotype O4, have repeatedly been isolated from diseased fish (Pazos, 1997; Santos *et al*., 1999; Cepeda and Santos, 2002;). However, we have recently isolated other filamentous gliding bacteria associated with disease in juveniles and adult stages of turbot and sole as well as from seawater. These microorganisms were usually isolated in mixed cultures with *T. maritimum*, *Vibrio pelagius*, and *V. splendidus*. The phenotypic characterization of these filamentous bacteria indicated that all of the strains were rather homogeneous in their biochemical and physiological reactions. Genetic analysis allowed us to demonstrate that these filamentous bacteria belong to the genus *Tenacibaculum* but are clearly differentiated from the other validly published species (unpublished results). In the present study the serological relationships amongst *Tenacibaculum* sp. isolated from fish and seawater was examined. The serological relationship of our *Tenacibaculum* sp. isolates with its closest phylogenetic neighbour *T. litoreum* and with *T. maritimum*, the only species of the Genus *Tenacibaculum* that include bacteria isolated from diseased fish, were also determined.

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3 – 4 November 2006, Athens, Greece, EU

## Material and methods

### Bacterial strains and phenotypic characterization

In the present work we used 17 strains isolated from diseased fish (4 strains) and seawater from fish holding tanks (13 strains) in the Norwest of Spain (Table 1). Reference strains of *Tenacibaculum maritimum* NCIMB2153 from the National Collection of Marine and Industrial Bacteria (NCIMB, Aberdeen, Scotland) and reference strain of *Tenacibaculum litoreum* JCM13039$^T$ from the Japaness Collection RIKEN BioResource Center, Hirosawa, Japan). The strains were routinely cultured on Marine agar (Cultimed, Spain) and incubated at 25ºC for 24-48h. Stock cultures of the strains were stored at -70ºC in Microbank™ tubes (Prolab Diagnostics, Ontario, Canada). The taxonomic position of the isolates was confirmed using morphological, physiological and biochemical tests following the procedures previously described (Bernardet *et al*., 2002).

### Antigens and antisera

Formalin killed cell (FKC) suspensions were prepared by fixation with 0.2% phosphate-buffered formalin. To obtain the thermostable "O" antigen, bacterial suspensions in phosphate-buffered saline (PBS, pH 7.2) were heated in boiling water for 1 h and washed once in the same saline solution. The antigens concentrations were adjusted to $10^9$ cells $ml^{-1}$ (McFarland standard nº 3).

Polyclonal antisera were produced against three representative strains of *Tenacibaculum* sp. isolated from turbot (R01.6.8.1), sole (LL04.11.1.1) and seawater (A37.1). Sera were obtained in rabbits as described by Sørensen and Larsen (1986) using formalin-killed cells as antigens. Whole cell antisera against the reference strain of *Tenacibaculum maritimum* NCIMB2153 was also obtained. Two kilograms New Zealand white rabbits were immunised intravenously at three days intervals with 0.2, 0.4, 0.8 and 1.0 ml of the appropriate formol-fixed bacteria adjusted to 1 x $10^9$ cells $ml^{-1}$ (McFarland standard nº 3). Two weeks later a booster inoculum of four injections (1.0 ml/dose) administered as before were given. Anaesthetised rabbits were bled by cardiac puncture eight days after the last injection. Sera recovered were aliquoted undiluted and frozen at -20ºC until use. Preimmune sera were previously collected from the rabbits.

### Serological characterization

Serological identification was performed using slide agglutination test as previously described (Santos *et al*., 1995). The test was carried out using both formalin-killed cells (FKC) and "O" antigens and rabbit whole cell antiserum raised against the strains previously indicated. Strong and rapid agglutination was registered as a positive result and no or only a weak agglutination that occurred after 1 to 2 min as a negative reaction. Auto-agglutination (spontaneous agglutination) control was carried out by mixing equal volumes of antigen suspension and sterile saline solution instead of antiserum.

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3 – 4 November 2006, Athens, Greece, EU

**Lipopolysaccharide and membrane protein preparations**

Whole-cell lysates of each isolate were prepared for protein analysis as previously described (Santos *et al*., 1995) and proteinase K treated samples for analysis of LPS were prepared as described by Hitchcock and Brown (1983).

Electrophoresis was carried out in commercial dodecyl sulfate-polyacrylamide 12% gel (SDS-PAGE) from Bio-Rad Laboratories, Richmond, Calif. with a Mini Protean I (Bio-Rad) system. Electrophoresis buffer was Tris-Glycine and the conditions were 100 min at 100 V. Standard protein markers were from Bio-Rad. Proteins were stained with Comassie brilliant blue R-250 (Bio-Rad), and LPS were stained with the silver stain kit (Bio-Rad).

**Immunoblotting analysis of protein and LPS**

For immunoblot assays fractionated antigens were electroblotted (at 30 V for 18 hours) from the gel onto 0,45-µm nitrocellulose paper (Bio-Rad) as described (Towbin *et al*., 1979). The nitrocellulose membranes were then blocked for 1h with 3% gelatin in Tris-buffered saline (TBS-3, pH 7.5), washed twice with TBS plus 0.05% Tween 20 (TTBS) and exposed to the antisera diluted 1:1000 in TBS containing 1% gelatin (TBS-1). LPS and protein components recognized by rabbit antisera were visualized by reacting the rinsed membranes with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) for 1 h. Reactions were developed using 0.1M carbonate buffer (pH 9.8) containing tetrazolium blue (0.3 mg $ml^{-1}$) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (0.15 mg $ml^{-1}$) was used.

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3 – 4 November 2006, Athens, Greece, EU

**Results and Discussion**

The results of slide agglutination using either the whole cell or thermostable somatic "O" antigens assays revealed that, regardless of their biochemical similarities, only 6 of 17 (35.3%) *Tenacibaculum* sp .strains tested were typeable being distributed into four serological O-groups (Table 1). Four strains isolated from seawater (A13.3, A35.1, A37.1 and A38.1) reacted with the serum anti-A37.1 and one strain (A52.1) reacted with the serum anti-R01.6.8.1. The strains R01.6.8.1and LL04.11.1.1 isolated from fish only reacted with their homologous antisera. The existence of intraspecific variability in serological characteristics have been previously reported in other bacteria isolated from marine fish culture system such us *T. maritimum* (Pazos, 1997, Santos *et al*., 1999; Avendaño-Herrera *et al*., 2004;), *Vibrio anguillarum* (Sørensen and Larsen, 1986; Austin *et al*., 1995; Santos *et al*., 1995), *Vibrio pelagius* and *V. splendidus* (Santos *et al*., 1996, 1997). Neither the *Tenacibaculum* sp strains isolated from fish or seawater nor the reference strain of *T.litoreum* JCM13039$^T$ reacted with the anti-*Tenacibaculum maritimum* NCIMB2153 serum. Negative reaction was also observed when reference strains of *Tenacibaculum maritimum* and *Tenacibaculum litoreum* were tested with sera anti-A37.1, anti- R01.6.8.1 and anti-LL0411.1.1. These results support that strains of filamentous bacteria analysed in the present study do not belong to the species *T. maritimum* or *T. litoreum*.

The SDS-analysis demonstrated that all strains present LPS with O-chains of variable length. Only slight differences were observed in the LPS profiles of strains belonging to different serological groups (data not shown). Therefore, at difference of that reported for other Gram-negative bacteria (Austin *et al*, 1995; Nomura and Aoki, 1985; Pyle and Schill, 1985; Toranzo *et al,* 1987; Santos *et al*, 1995), it would appear that serotypes of *Tenacibaculum* sp isolates established on the basis of slide agglutination do not coincide exactly with LPS profiles.

The electrophoretic analysis of total proteins showed the existence of homogeneity among the *Tenacibaculum* sp isolates with common bands between 20 and 95 kDa (data not shown). The reference strain of *T. maritimum* NCIMB2153 showed a very similar protein pattern. The lack of agreement between the composition of serogroups established by agglutination and grouping resulting from examination of the membrane protein profiles observed in the present study was also described in *Vibrio anguillarum* by Austin *et al.* (1995).

Immunoblot analysis of LPS and proteins demonstrated that sera against the *Tenacibaculum* sp. strain R01.6.8.1, LL04.11.1.1 and A-37.1 do not react with the cellular antigens from the reference strain of *T. maritimum* or from *Tenacibaculum* sp. from other serological groups, corroborating the existence of serological heterogeneity among *Tenacibaculum* species isolated from fish culture system observed using slide agglutination assay. The Fig. 1 shows the results of immunoblot assays using LPS as antigen and antisera against the strain isolated from seawater A37.1 (A) and the reference strain of *T. maritimum* NCIMB 2153 (B).

In conclusion, the overall results indicated that strains of filamentous bacteria isolated from turbot and sole culture systems are serologically heterogeneous and can be typed on the basis of their different "O" antigen.

**Acknowledgments**

This investigation was supported by grant PGIDIT 04 RMA 003E from the Xunta de Galicia, Spain. The authors are indebted to the NCMIB and RIKEN BRC for providing the reference strains of *Tenacibaculum maritimum* and *Tenacibaculum litoreum*, respectively.

3rd International Congress on Aquaculture, Fisheries Technology and Environmental Management
3 – 4 November 2006, Athens, Greece, EU

Table 1.- Results of slide agglutination with whole cells (FKC) and "O" antigens.

| Strains | Origin | Antigen | Anisera against | | | |
|---|---|---|---|---|---|---|
| | | | NCIMB2153 | R01.6.8.1 | LL04.11.1.1 | A37.1 |
| Reference *T. maritimum* | | | | | | |
| NCIMB2153 | *Acanthopagrus schlegeli*, Bleeker, Japan | FKC | + | – | – | – |
| | | "O" | + | – | – | – |
| *T. litoreum* | | | | | | |
| JCM130309T | Tidal flat sediment, Korea | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| Present study | | | | | | |
| R01.6.8.1 | *S. maximus*, L, Spain | FKC | – | + | – | – |
| | | "O" | – | + | – | – |
| LMO2.4.4.2 | *Solea senegalensis*, Kaup, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| LLO4.11.1.1 | *S. senegalensis*, Kaup, Spain | FKC | – | – | + | – |
| | | "O" | – | – | + | – |
| LLO4.12.1.7 | *S. senegalensis*, Kaup, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A13.3 | Seawater, Spain | FKC | – | – | – | + |
| | | "O" | – | – | – | + |
| A22.2 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A35.1 | Seawater, Spain | FKC | – | – | – | + |
| | | "O" | – | – | – | + |
| A35.2 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A36.1 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A37.1 | Seawater, Spain | FKC | – | – | – | + |
| | | "O" | – | – | – | + |
| A38.1 | Seawater, Spain | FKC | – | – | – | + |
| | | "O" | – | – | – | + |
| A41.1 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A42.1 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A42.2 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A51.1 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |
| A52.1 | Seawater, Spain | FKC | – | + | – | – |
| | | "O" | – | + | – | – |
| A61.1 | Seawater, Spain | FKC | – | – | – | – |
| | | "O" | – | – | – | – |

NCIMB, National Collection of Marine and Industrial Bacteria (Aberdeen, Scotland)
JCM, Japaness Collection (RIKEN BioResource Center, Hirosawa, Japan).

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3 – 4 November 2006, Athens, Greece, EU

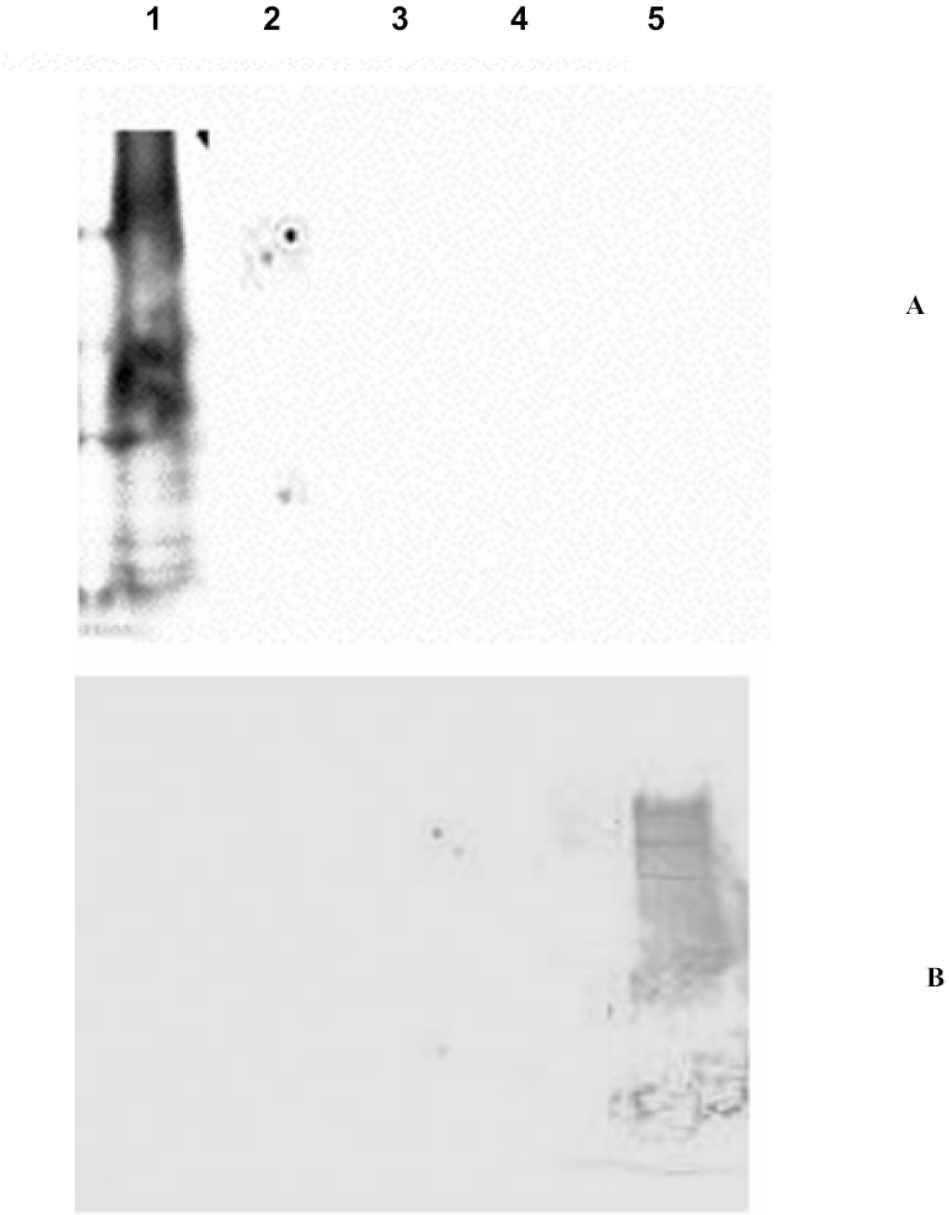


Fig 1.- Immunoblot of LPS of filamentous bacteria used in the present study using sera anti-NCIMB2153 (A) and anti-A37.1 (B). Lanes 1, NCIMB2153, 2, R016.8.1, 3, A42.2, LL04.11.1.1, A37.1.

3rd International Congress on Aquaculture, Fisheries Technology and Environmental Management
3 – 4 November 2006, Athens, Greece, EU

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**Artículo nº7.** Piñeiro-Vidal, M. & Santos, Y. (2008).Identification of immunogenic antigens of the fish pathogens *Tenacibaculum gallaicum*, *T. discolor* and *T. soleae*. *Submitted.*

# Identification of immunogenic antigens of the fish pathogens *Tenacibaculum gallaicum*, *T. discolor and T. soleae*

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Running title : Antigens of *Tenacibaculum gallaicum T. discolor* and *T. soleae*

**Abstract**

This study was focussed on the serological characterization of strains of the species *T. gallaicum*, *T. discolor* and *T. soleae* isolated from turbot and sole culture systems and in the identification of the dominant inmunogens present in their cell envelope and extracellular products (ECP). SDS-PAGE and immunoblot assays of cell envelope and ECP components demonstrated the existence of antigenic heterogeneity within *T. gallaicum*, *T. discolor* and *T. soleae* species and the lack of immunological relationship among these species. Moreover, turbot antiseum against formalin inactivated whole cell of strain LPV 1.7 of *T. maritimum* showed a positive reaction with the homologous cell envelope LPS and proteins but did not recognize the cell envelope preparations obtained from the type strains of *T. discolor*, *T. gallaicum* and *T. soleae*.

**Keywords:** *Tenacibaculum*, extracellular products, protein, lipopolysaccharide, immunoblot, fish.

## Introduction

Marine flexibacteriosis is, at present, one of the main pathological problems for the culture of turbot (*Psetta maxima*, L.) and sole (*Solea senegalensis*, Kaup.) cultured in Spain (Pazos et al., 1993, Piñeiro-Vidal et al., 2007). Affected fish usually showed the typical signs described for marine flexibacteriosis (eroded mouth, rotten fins, shallow skin lesions and paleness of internal organs). Although *Tenacibaculum maritimum* is the main causative agent of this disease, other filamentous gliding bacteria pathogenic for fish and belonging to the new described species *T. gallaicum*, *T. discolor* and *T. soleae* have been isolated from diseased fish either immunized against *T. maritimum* or unvaccinated and/or from seawater in fish-holding tanks (Piñeiro-Vidal et al., 2007; Piñeiro-Vidal et al., 2008 a, Piñeiro-Vidal et al., 2008 b). These microorganisms showed similar phenotypic characteristics that *T. maritimum* but can be differentiated on the basis of PCR amplification of genomic DNA by using oligonucleotide species-specific primers and by slide agglutination test (Piñeiro-Vidal et al., 2007), as well by cellular fatty acids analysis (Piñeiro-Vidal et al., 2008 c). In addition, serological studies using immunoblot assays demonstrated that isolates of the species *T. gallaicum* and *T. discolor* were antigenically different from *T. maritimum* (Piñeiro-Vidal et al., 2006), suggesting that vaccination against *T. maritimum* do not necessarily will protect fish against the other fish pathogenic *Tenacibaculum* species. Previous studies have indicated the existence of serological heterogeneity within *T. maritimum* strains isolated from different fish species and geographic areas (Pazos et al., 1993; Pazos, 1997; Santos et al., 1999; Avendaño-Herrera et al., 2004; Avendaño-Herrera et al., 2005). However, similar studies have not been carried out with the other described fish pathogenic *Tenacibaculum* species. This work reports on the antigenic characterization of *T. gallaicum*, *T. discolor* and *T. soleae* strains isolated from turbot and sole culture systems.

## Material and methods

### Bacterial strains and phenotypic characterisation

In the present study we used fifteen strains of the species *Tenacibaculum gallaicum* (9 strains), *T. discolor* (5 strains), and *T. soleae* (1 strain) isolated from diseased cultured turbot (*Psetta maxima*) and sole (*Solea senegalensis*) and from seawater in fish-holding tanks. The type strains DSM 18841 of *T. gallaicum*, DSM 18842 of *T. discolor* and NCIMB 14368 of *T. soleae,* and the strain LV1.7 of *T. maritimum* were also included (Table 1). Stock cultures of the strains were stored at -70ºC in Microbank™ tubes (Prolab Diagnostics, Ontario, Canada) until passage on *Flexibacter maritimus* Medium (FMM) agar (Pazos et al., 1996). All bacterial cultures were incubated at 25°C for 48h. The taxonomic status of isolates was confirmed using standard morphological, biochemical, and physiological tests as previously described (Piñeiro-Vidal et al., 2007; Piñeiro-Vidal et al., 2008a, b). The serological characterization was carried out by slide agglutination using formalin killed whole cells as antigen and rabbit polyclonal sera against the type strains of *T. gallaicum*, *T. discolor* and *T. soleae*.

### Production of antisera

Antisera against formalin killed whole cells of type strains of *Tenacibaculum gallaicum* (DSM 18841), *T. discolor* (DSM 18842) and *T. soleae* (NCIMB 14368) were produced following the procedure described by Sørensen & Larsen (1986). Briefly, bacterial cultures were harvested from FMM agar plates in sterile phosphate buffered saline (PBS, pH 7.2) with formaldehyde added to a final concentration of 0.3%. Inactivated cells were washed three times in sterile PBS by centrifugation (3000*xg*, centrifuge Sigma 3K30, Postfach, Osterodeam Harz, Germany), resuspended in the same solution to a concentration of $10^9$ cells/ml (tube 4 MacFarland) and stored at 4ºC until use. Polyclonal antisera to these

strains were raised in New Zealand White rabbits by intravenous injection of antigen suspension administered at two days intervals in doses of 0.2, 0.4, 0.8, and 1.0 ml. Two weeks later the immunization procedure was repeated by intravenous injection of 1.0 ml of antigen suspension. One week after the last injection, the blood was collected by cardiac puncture, allowed to clot overnight at 4ºC and the recovered sera were stored at -20ºC until use. In order to evaluate if turbot inmmunized aginst *T. maritimum* are cross-protected against tenacibaculosis caused by the other pathogenic *Tenacibaculum* species, serum against formalin inactivated whole cells of the strain of *T. maritimum* LPV 1.7 were raised in turbot (500 g body weight) following the procedures described by Hasting and Ellis (1988).

**Membrane protein and lipopolysaccharide (LPS) extraction**

The cell envelope proteins and LPS of the *Tenacibaculum* strains were obtained from cultures on agar FMM. Briefly, bacteria were resuspended in 3 ml of 10 mM Tris buffer (pH 8.0) containing 0.3% NaCl and sonicated with a Labsonic Sonifier (Sartorius Stedim Biotech S.A. Aubagne, France), (100 W, 60 s, four times). After centrifugation at 10,000 x*g* for 5 min, to eliminate possible intact cells, the supernatants were transferred to new tubes and centrifuged again for 60 min at 30,000x*g* at 4ºC. The resultant precipitates were resuspended in sterile distilled water and kept at -30ºC until use. Protein concentration was determined by the procedure of Bradford (Bradford, 1976) and adjusted to 2mg/ml. LPS were obtained from whole-cell lysates following basically the method of Hitchcock and Brown (1983). Whole-cell lysates were boiled at 100ºC for 10 min and treated at 60ºC for 1 hour with a solution of proteinase K (1mg/ml) in sterile distilled water.

**Extraction of extracellular products**

The extracellular products (ECP) from a group of strains representative of *T. gallaicum* and *T. discolor* species were obtained by the cellophane plate technique as previously decribed (Santos et al., 1991). Briefly, sterilized cellophane sheets were placed on the surface of FMM plates and inoculated by spreading 0.5 ml of a broth culture of each strain with a sterile swab. After 48h of incubation at 25ºC, cells were washed off the cellophane with PBS. The cell suspension were centrifuged at 10,000xg for 30 min at 4ºC and the resulting supernatants were filtered (0.45µm Millipore membranes) and stored at –30ºC. LPS from ECP were extracted by mixing aliquots of 10 min boiled ECP with the proteinase K solution. The mixture was incubated as for extraction of LPS from bacteria.

**SDS-PAGE electrophoresis**

The LPS and proteins from cell envelope and ECP were analyzed by SDS-PAGE (Laemmli, 1970) in the Bio-Rad Mini Protean II slab cell system. Protein and LPS samples were mixed (1:1) with 2x electrophoresis final sample buffer (FSB) (5ml Tris-HCl 0.25M pH=6.8, 0.4g SDS, 2ml glycerol, 0.2mg bromophenol blue and 2-mercaptoethanol), heated 10 min at 100ºC and electrophoresed at 150 V through a 12.5% acrylamide gel. Gel and buffer compositions were as recommended in the Mini Protean II slab cell instruction manual. Pre-stained molecular mass markers (Bio-Rad) were used as controls. Proteins were stained with Comassie brilliant blue R-250 (Bio-Rad) and LPS were silver stained by using the Silver stain plus system (Bio-Rad).

**Immunoblot analysis using rabbit antisera**

Prior to immunostaining, protein and LPS antigens were transferred from polyacrylamide gels to nitrocellulose membranes as described by Towbin et al. (1979). Antigens were

electroblotted at 0.1 mA for 18 h in Tris-Glycine-Methanol transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20% methanol). After transfer, the nitrocellulose membranes were blocked for 1 h with 3% gelatine in Tris-buffered saline (TBS) (pH 7.5), washed twice with TBS plus 0.05% Tween 20, and incubated for 1 h in control or diluted immune rabbit serum in TBS containing 1% gelatine. The membranes were again washed with TBS-0.05% Tween 20 and then incubated for 1 hour with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) diluted in TBS-1% gelatine. Bands were visualised by incubating membranes in 0.1 M carbonate buffer (pH 9.8) containing tetrazolium blue (0.3 mg/ml) and 5-bromo-4-chloro-3-indolylphophate toluidine salt (0.15 mg/ml).

**Immunoblot analysis of cell envelope compounds immunogenic for turbot**

For immunoblot assays fractionated antigens were electroblotted to nitrocellulose membranes and then exposed to the polyclonal turbot antiserum against whole cell of strain LPV 1.7 of *T. maritimum*. Cell envelope components recognized by the turbot antiserum were visualized by reacting the membranes with rabbit anti-turbot IgM for 3 h and with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) for 1 h. Reactions were developed as above described.

## Results

### Taxonomic characterization of the strains

On the basis of morphological, biochemical and physiological characteristics the strains were identified as *T. gallaicum* (10 strains), *T. discolor* (6 strains) and *T. soleae* (2 strains). The slide agglutination assay demonstrated that *T. gallaicum*, *T. discolor*, and *T. soleae* strains only react with the antisera raised against their respective type strains with no cross reaction being detected (Table 2).

### Characterisation of cell envelope components

The membrane protein profiles of a representative group of strains of *Tenacibaculum gallaicum*, *T. discolor*, and *T. soleae* were analyzed by SDS-PAGE and immunoblot. All strains showed a high range of protein bands between 170 and 10 kDa. The protein patterns of the strain of *T. gallaicum* (Fig 1a) were almost identical and very similar to those of the strains of *T. discolor* (Fig 2a) and *T. soleae* (data not shown). The profiles of *T. gallaicum* and *T. discolor* were characterized by the presence of two major protein compounds of approximately 60 and 50 kDa. Three major protein compounds of 70, 45 and 22 kDa were observed in the *T. soleae* strains analyzed (data not shown). Western blotting analyses showed that rabbit sera contained antibodies that reacted with several components of the cell envelope preparations. Three to four immunodominant proteins were observed in the strains analyzed (Figs 1b, 2b and 3). Moreover, it was found that *T. gallaicum* isolates (Fig 1b) were immunologically heterogeneous with regard to cell envelope proteins while *T. discolor* (Fig 2b) and *T. soleae* (Fig 3) strains were more homogeneous. LPS of representative strains of each *Tenacibaculum* species used in the study were visualized by immunostaining with the antisera against the respective type strains (anti-*T. gallaicum* DSM 18841, anti-*T. discolor* DSM 18842 and anti-*T. soleae* NCIMB 14368). The LPS banding pattern obtained for the strains of the three species can be separated into two

fractions of high (near to the top of the gel) and low (near the bottom of the gel) mobility (Figs 1c, 2c and 4c). Only the LPS from some of the strains representatives of each species analyzed reacted with antiserum against the type strains, indicating the existence of antigenic heterogeneity within *T. gallaicum*, *T. discolor* (Figs 1c, 2c) and *T. soleae* species. Moreover, LPS samples from the type strain of *T. gallaicum* were not stained with the antisera against the type strains of *T. discolor* and *T. soleae* (Figs 4b and 4c) but reacted positively with the homologous antiserum (Fig 4a). Similarly, LPS samples from the type strains of *T. discolor* and *T. soleae* only reacted with their homologous antisera (Fig 4b and 4c).

**Analysis of extracellular antigens**

The SDS-PAGE analysis of the ECP from representative strains of *T. gallaicum* and *T. discolor* showed the existence of different protein and LPS profiles (data not shown). The molecular masses of the ECP protein ranged from 15 to 130 kDa. The LPS present in the ECP showed a banding pattern similar from those obtained with the LPS from cell envelopes. Serum obtained against whole cell of *T. gallaicum* DSM18841 and *T. discolor* DSM 18842 strongly reacted with the high-molecular-weight zone of the extracellular LPS and with some proteins obtained from strains of the same species (Fig 5 a, b and 6a and 6b).

**Immunoblot assays using turbot antiserum**

Immunoblotting analyses showed that turbot antiseum against whole cell of the strain LPV 1.7 of *T. maritimum* showed a strong reaction with the homologous cell envelope protein

and a weak reaction with the LPS. No reaction was observed with the cell envelope preparations obtained from the type strains of *T. discolor*, *T. gallaicum* and *T. soleae*.

**Discussion**

This study was focused in the serological characterization of a group of strains belonging to the species *T. gallaicum*, *T. discolor* and *T. soleae* isolated from turbot and sole culture systems and in the identification of the dominant antigens present in their cell envelope and extracellular products. The results of slide agglutination assay demonstrated that *T. gallaicum*, *T. discolor* and *T. soleae* were serologically unrelated. In a previous study we have also demonstrated the lack of serological relationship of these microorganisms with *T. maritimum*, other fish pathogenic species of the genus *Tenacibaculum* (Piñeiro-Vidal et al., 2006).

The electrophoretic analysis of cell envelope components showed that, regardless of their isolation source, the strains belonging to the species *T. gallaicum*, *T. discolor* and *T. soleae* possessed very similar protein patterns. Moreover, strains of the three species contained a large number of apparently similar proteins. The existence of similarity in the cell envelope protein profiles has also been described in *Tenacibaculum maritimum* (Pazos et al., 1993; Bernardet et al., 1994; Pazos, 1997; Avendaño-Herrera et al., 2004). Nevertheless, immunoblot assays demonstrated that only *T. discolor* and *T. soleae* constituted antigenically homogeneous species with regard to cell envelope proteins. In addition we have demonstrated that cell envelope proteins from *T. gallaicum*, *T. discolor* and *T. soleae* are immunologically unrelated. On the other hand, we have observed that the LPS banding pattern of *T. gallaicum*, *T. discolor* and *T. soleae* were characterized by the presence of two fractions of high and low molecular-weight, in contrast with the ladder-like structure described for *T. maritimum* (Pazos, 1997, Avendaño-Herrera et al., 2004, Piñeiro-Vidal et

al., 2006). The differences in the profiles of LPS of *T. maritimum* from the LPS of the other three fish pathogenic *Tenacibaculum* species analyzed in this work could be used to development of method for the rapid and specific detection of these pathogens. The immunoblot analysis of LPS also demonstrated the existence of antigenic heterogeneity within *T. gallaicum*, *T. discolor* and *T. soleae* species and the lack of antigenic relationship among the bacterial cell envelope LPS of these species. The immunoblot analysis of ECP components using serum obtained against whole cells of *T. discolor* DSM 18842 and *T. gallaicum* DSM 18841 indicated that LPS and protein from cell envelope and extracellular products are immunologically related. These results are in agreement with that found in *Listonella anguillarum* (Evelyn, 1984; Santos et al 1995). When the cell envelope components from type strains of the species *T. discolor*, *T. gallaicum* and *T. soleae* were analyzed using turbot antiserum against the strain LPV 1.7 of *T. maritimum*, no reaction was observed, indicating that turbot vaccinated against *T. maritimum* are not protected agisnt tenacibaculosis caused by the other fish pathogenic *Tenacibaculum* species. Future studies will investigate whether LPS and/or protein from cell envelope and/or ECP should be included in the formulation of effective tenacibaculosis vaccines.

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Table 1. Bacterial strains used in this study.

| **Strains** | **Species** | **Source** | **Geographical origin** |
|---|---|---|---|
| LPV 1.7 | *Tenacibaculum maritimum* | Turbot (*Psetta maxima*, L.) | Galicia (NW of Spain) |
| DSM 18841 | *Tenacibaculum gallaicum* | Fish holding tanks | Galicia (NW of Spain) |
| A13.3 | *T. gallaicum* | Fish holding tanks | Galicia (NW of Spain) |
| A35.1 | *T. gallaicum* | Fish holding tanks | Galicia (NW of Spain) |
| A37.1 | *T. gallaicum* | Fish holding tanks | Galicia (NW of Spain) |
| A38.1 | *T. gallaicum* | Fish holding tanks | Galicia (NW of Spain) |
| A71.3 | *T. gallaicum* | Fish holding tanks | Galicia (NW of Spain) |
| CLT07.1 | *T. gallaicum* | Turbot (*P. maxima*, L.) | Galicia (NW of Spain) |
| R04 5.0.1 | *T. gallaicum* | Turbot (*P. maxima*, L.) | Galicia (NW of Spain) |
| RO06.0.1 | *T. gallaicum* | Turbot (*P. maxima*, L.) | Galicia (NW of Spain) |
| RO06.0.3 | *T. gallaicum* | Turbot (*P. maxima*, L.) | Galicia (NW of Spain) |
| DSM 18842 | *Tenacibaculum discolor* | Sole *(Solea senegalensis*, Kaup) | Galicia (NW of Spain) |
| LC06 11.5.1 | *T. discolor* | Sole (*S. senegalensis*, Kaup) | Galicia (NW of Spain) |
| LC 07 3.2.2 | *T. discolor* | Sole (*S. senegalensis*, Kaup) | Galicia (NW of Spain) |
| R01 6.8.1 | *T. discolor* | Turbot (*P. maxima*, L.) | Galicia (NW of Spain) |
| A22.2 | *T. discolor* | Fish holding tanks | Galicia (NW of Spain) |
| A36.1 | *T. discolor* | Fish holding tanks | Galicia (NW of Spain) |
| NCIMB 14368 | *Tenacibaculum soleae* | Sole *(S. senegalensis*, Kaup) | Galicia (NW of Spain) |
| A71.2 | *T. soleae* | Fish holding tanks | Galicia (NW of Spain) |

**FIGURE LEGENDS**

Fig. 1- SDS-PAGE (A) and immunoblot of membrane proteins (B) and LPS (C) from *T. gallaicum* using rabbit antiserum against *T. gallaicum* DSM 18841. Lanes: a, DSM 18841; b, A71.3; c, RO06.0.1; d, RO06.0.3; e, R04 5.0.1; f, CLT07.1; MWM, molecular weight marker. Numbers indicated molecular weight expressed in Kd.

Figure 2.- SDS-PAGE (A) and immunoblot of membrane proteins (B) and LPS (C) from *T. discolor* using rabbit antiserum against *T. discolor* DSM 18842. Lanes: a, A22.2; b, R01 6.8.1; c, DSM 18842; d, LC06 11.5.1; e, LC07 3.2.2; MWM, molecular weight marker. Numbers indicated molecular weight expressed in Kd.

Figure 3.- Western blot analysis of membrane proteins from *Tenacibaculum* sp. using the antiserum obtained in rabbit against the complete cell of *Tenacibaculum soleae* strain NCIMB 14368. Lanes: a, NCIMB 14368; b, A71.2; MWM, molecular weight marker. Numbers indicated molecular weight expressed in Kd.

Figure 4.- Western blot analysis of LPS from *Tenacibaculum* sp. using the antiserum obtained in rabbit against the complete cell of *Tenacibaculum gallaicum* type strain DSM 18841(A), *T. discolor* type strain DSM 18842 (B) and *T. soleae* type strain NCIMB 14368 (C). Lanes: a, DSM 18841; b, DSM 18842; c, NCIMB 14368.

Figure 5.- Immunoblot of proteins (a) and LPS (b) present in the ECP from *T. gallaicum*. Lanes: a, RO06.0.3; b, RO06.0.1; c, A13.3; d, DSM 18841; MWM, molecular weight marker. Numbers indicated molecular weight expressed in Kd.

Figure 6.- Immunoblot of proteins (a) and LPS (b) present in the ECP from *T. discolor*. Lanes: a, A22.2; b, LC07 3.2.2; c, DSM 18842; d, R01 6.8.1; MWM, molecular weight marker. Numbers indicated molecular weight expressed in Kd.

Figure 7. Immunoblot of LPS present in the cell wall from *T. maritimum*, *T. gallaicum*, *T. discolor*, and *T. soleae* using antiserum raised in turbot against the strain of *T. maritimum* LPV1.7. Líneas: a, *Tenacibaculum maritimum*, LPV1.7; b, *T. gallaicum*, DSM 18841; c, *T. discolor*, DSM 18842; d, *T. soleae*, NCIMB 14368; MWM, marcador de masa molecular. Los números indican la masa molecular en Kdaltons.

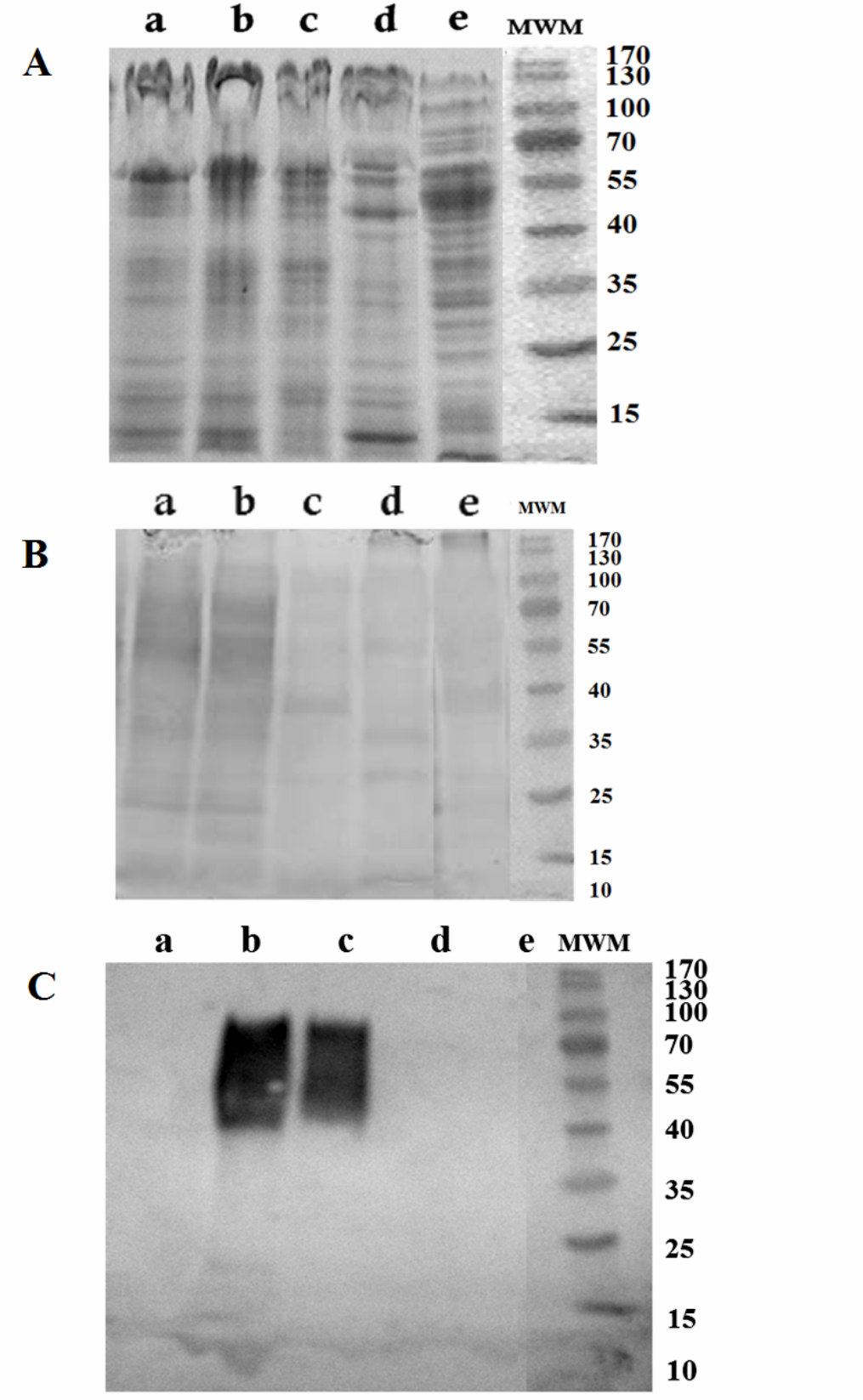
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Fig 1

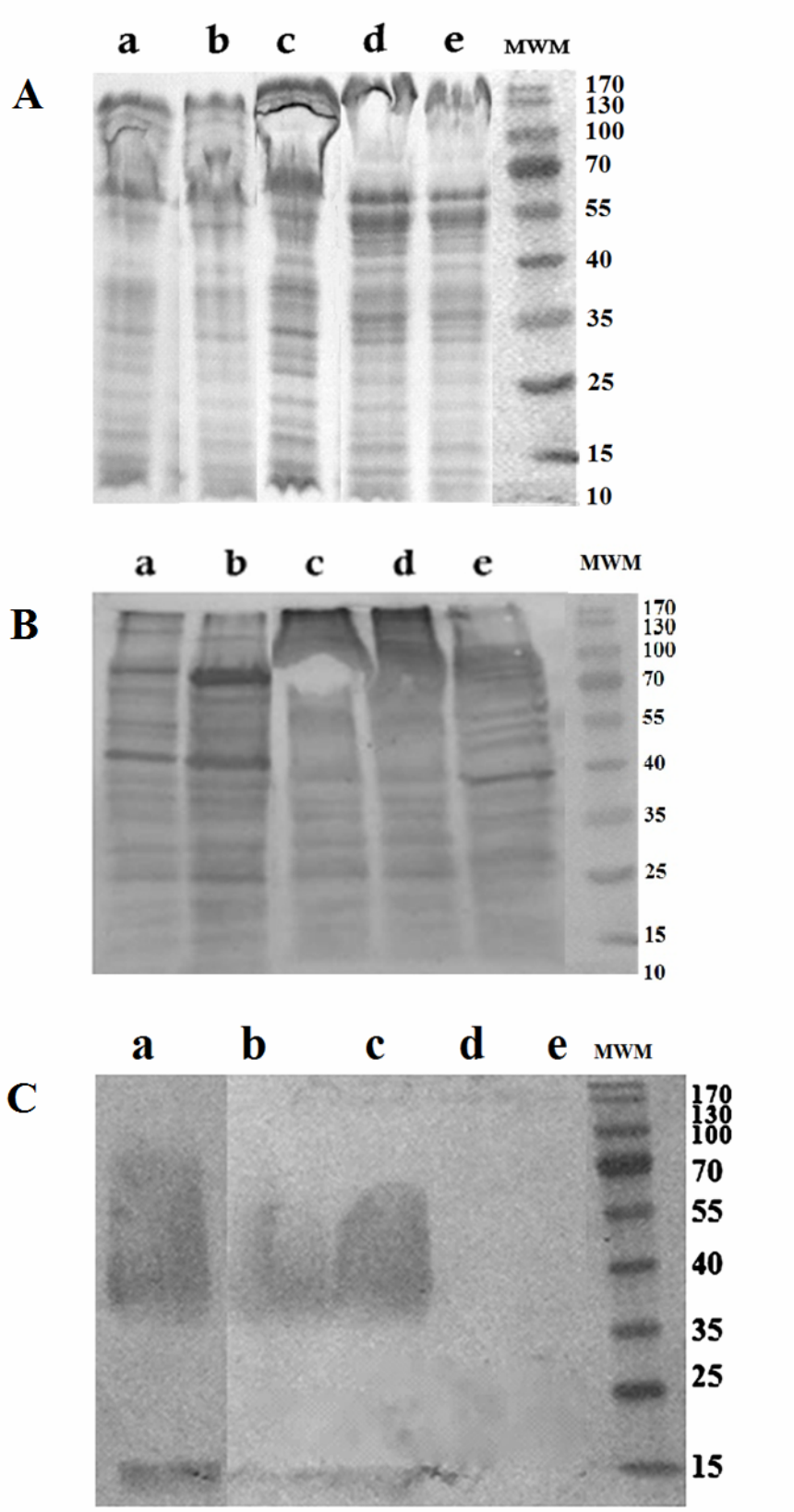
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Fig. 2

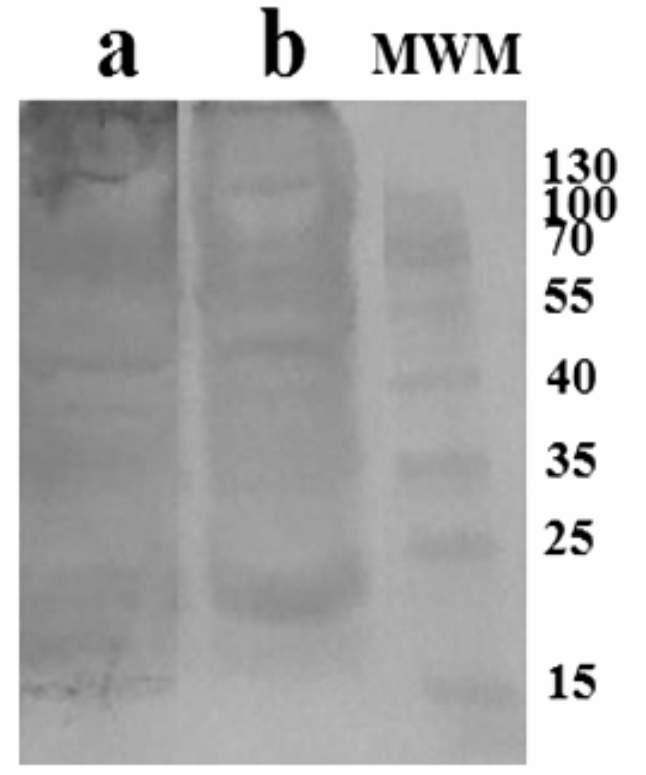


Fig. 3

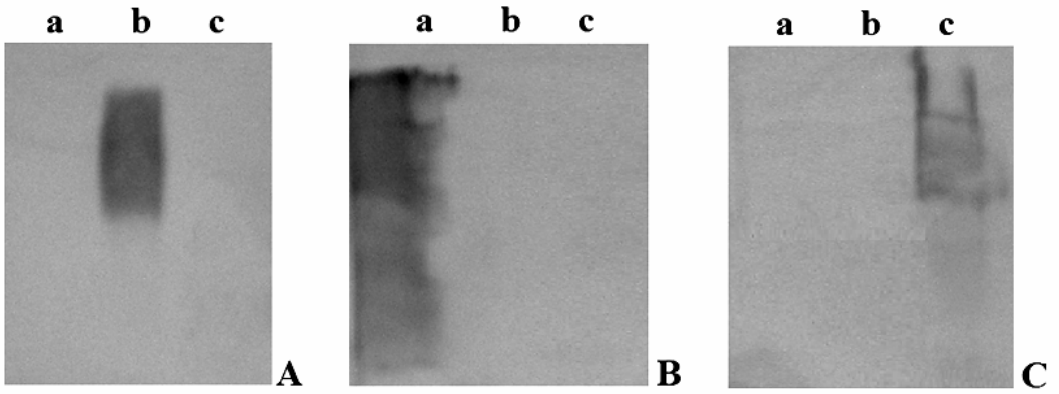


Fig 4.

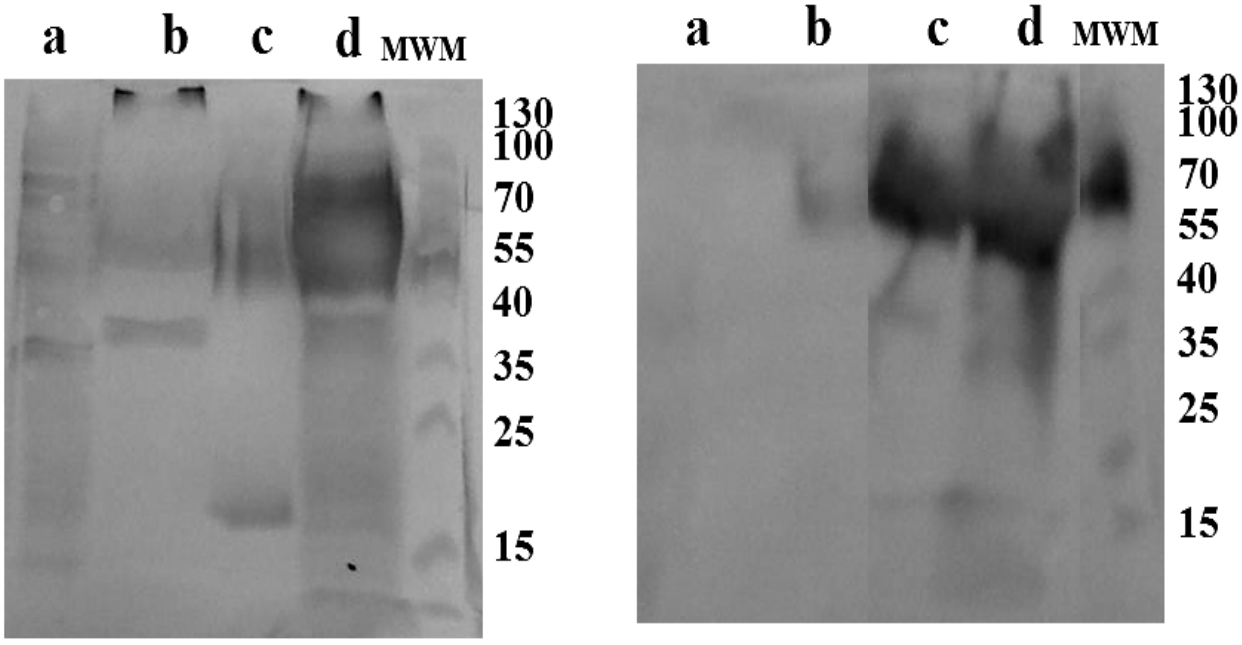
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Fig .5

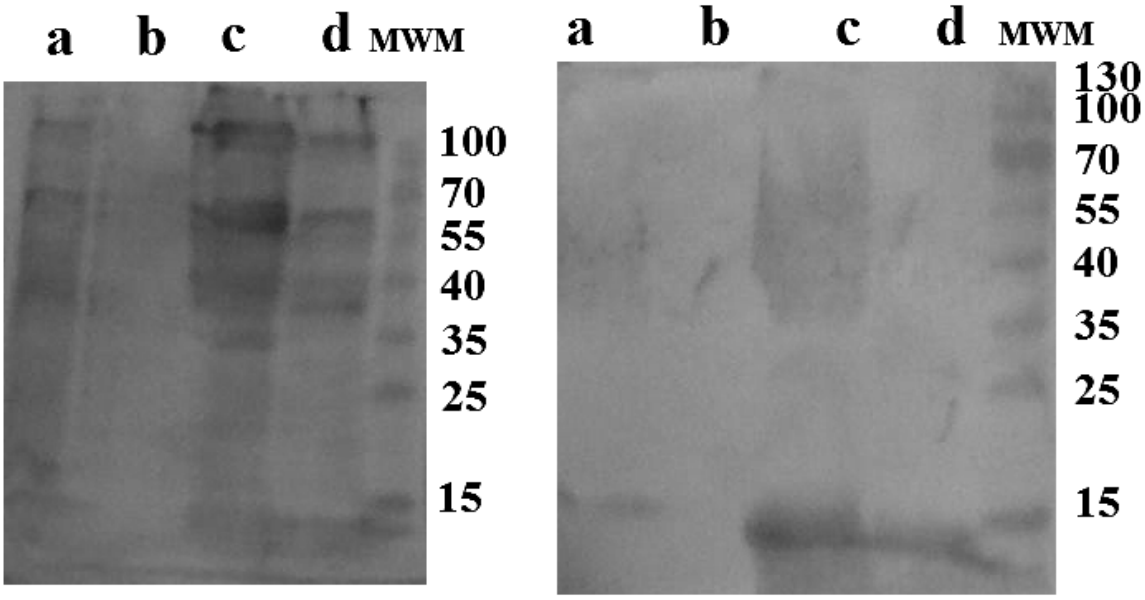


Fig. 6.-

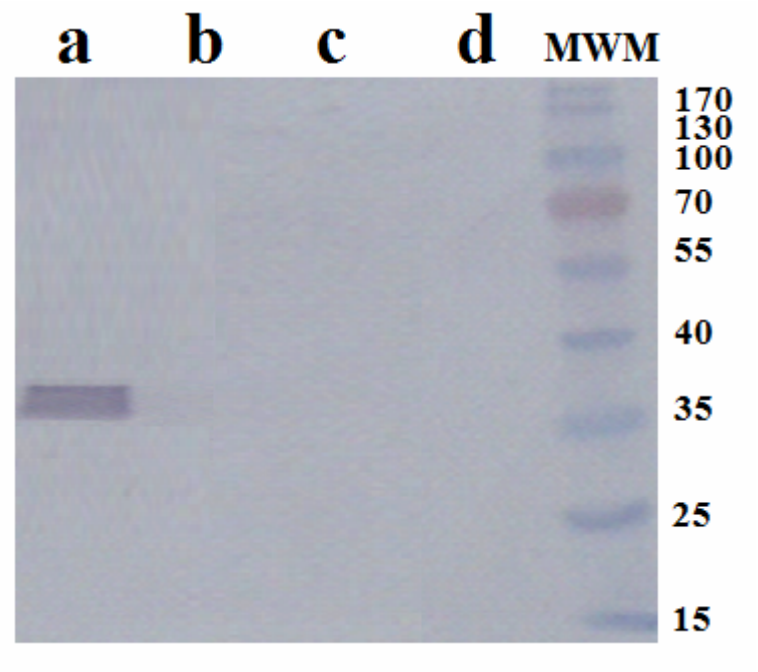


Fig 7

# Capítulo VI Detección y caracterización de señales de *quorum sensing* en *Tenacibaculum discolor*

# VI . Publicaciones

**Artículo nº 8.** Piñeiro-Vidal, M., Romero, M., Cámara, M., Seoane, R., Santos, Y. & Otero, A. (2008) Profiling of acylated homoserine lactones of *Tenacibaculum discolor* "in vitro" and "in vivo". (Manuscrito en preparación).

# Profiling of acylated homoserine lactones of *Tenacibaculum discolor* "in vitro" and "in vivo"

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Running title: Profiling of AHLs in *T. discolor*

Submitted to: (manuscript in preparation)

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**Abstract**

This work describes the "in vitro" and "in vivo" production of acylated homoserin lactones by the strain DSM 18842 of the fish pathogenic *Tenacibaculum discolor* species. The HPLC-MS analysis unequivocally identified the *N*-butyryl-L-homoserine lactone (C4-HSL) as the only QS signal present in the acidified extracts of "in vitro" cultures of the strain DSM 18842 of *T. discolor* under the culture conditions tested. The same compound was also detected in the internal organs (kidney, spleen, and liver) of asymptomatic, moribund and dead experimentally infected fish. However, HPLC-MS analysis demonstrated quantitative differences in the amount and kinetic of AHL produced by *T. discolor* in FMM and MB media. Treatment of turbot head-kidney leucocytes with C4-HSL did not affect their respiratory burst activity. In conclusion, *Tenacibaculum discolor* produces a single AHL, C4-HSL at detectable leves both "in vitro" and "in vivo"during experimental infection of turbot, which may play a role in the pathogenesis of the tenacibaculosis caused by this species.

Keywords: quorum sensing, AHL's, flexibacteriosis, *Tenacibaculum discolor*, *Flavobacteriaceae.*

## Introduction

In lasts years cell-cell communication mediated by acylated homoserine lactones (AHLs) signals was described in many gram-negative bacteria (Whitehead et al., 2001; Manefield et al., 2004; Bruhn et al., 2005; Milton, 2006; Romero et al., 2008). These molecules that are secreted to the culture media are involved in a cell-density dependent process of regulation of gene expression know as quorum sensing (QS) (Williams et al, 2000, Donabedian, 2003; Williams et al., 2007). QS signals or auto inducers have an important role in the expression of virulence mechanisms required to colonize skin and intern tissues of organisms affected of determined disease (Milton et al., 1997; Swift et al., 1999; Greenberg, 2000; Buch et al., 2002; Milton, 2006). AHLs may also modulate host immune response to invading pathogens (Williams et al, 2000, Donabedian, 2003; Williams et al., 2007). Among Gram-negative bacteria capable of causing infections in human, homoeothermic animals, and plants, AHL-dependent quorum-sensing circuits have been described in *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *B. pseudomallei*, *Serratia marcescens*, *Erwinia carotovora*, *Rhizobium leguminosarum*. In many obligate Gram-negative human pathogens (*Neisseria meningitidis*, *Haemophilus influenzae*, *Helicobacter pylori*) not AHLs signals have been detected (Williams et al, 2000, Donabedian, 2003; Williams et al., 2007). Others like *E. coli* or *Salmonella typhimurium* do not produce the signals, but possess a gene homologous to the receptor LuxR that confers the capacity of sensing the signals produced by other bacteria (Turovskiy et al., 2007). AHL-mediated QS systems have been also described in fish pathogens of the species *Vibrio harveyi, V. fischeri, Listonella anguillarum, Aeromonas hydrophila*, *A.*

*salmonicida* and *Yersinia ruckeri* (Buch et al., 2003; Buchholtz et al., 2006; Bruhn et al., 2005; Manefield et al., 2000). There are evidences that expression of some virulence factors (protease, siderophore, toxins) and biofilm formation are AHL-regulated in some of these fish pathogenic bacteria (Swift et al., 1997; 1999; Lilley and Bassler, 2000; Manefield et al., 2000). Moreover, the detection of AHLs in the tissues of fish infected with *L. anguillarum* (Buch et al, 2003; Buchholtz et al., 2006) and *Y. ruckeri* (Kastbjerg et al., 2006) indicates that quorum sensing plays a regulatory role in the infectious processes. Due to the prevalence of quorum sensing systems among fish pathogens, the inhibition of these processes has been proposed as an alternative to the use of antibiotics in aquaculture, which are leading to the rapid development of resistance (Defoirdt et al., 2004). Several results indicate the viability of this approach for the treatment of infections in aquaculture. Mortality of rainbow trout infected with *L. anguillarum* was significantly reduced when treated with a furanone, a quorum sensing inhibitor (Rasch et al, 2004). In *V. harveyi*, a fish pathogen that produces and responds to three different autoinducers: an AHL (*harveyi* autoinducer, HAI-1), AI-2 and one still uncharacterized signal named CAI-1 (Henke and Bassler, 2004), the inactivation of the two first quorum sensing channels significantly increased growth rate of the rotifer *Brachionus plicatilis* (Tinh et al., 2006) and survival in *Artemia franciscana* (Defoirdt et al., 2005) in challenges carried out with this pathogen under gnotobiotic conditions, although differing regarding the relative importance of both quorum sensing channels in the control of virulence traits (Tinh et al., 2006). On the other hand the mutation of the quorum sensing system in *A. hydrophila*, in which AHLs control important

virulence factors such as biofilm formation and exoprotease production, did not affect its virulence towards *A. franciscana* (Defoirdt et al., 2005).

Recently, Romero et al. (2008) described the presence of AHL activity (C4-HSL, C8-HSL, C12-HSL and C14-AHLs) in the culture media of different strains of *Tenacibaculum maritimum*, a member of the Family *Flavobacteriaceae* [*Cythofaga-Flavobacterium-Bacteroides* (CFB) cluster], that is considered the main causative agent of tenacibaculosis in cultured and wild marine fish (Bernardet et al, 1997; Santos et al., 1999). This is the first report of the production of AHLs outside the Proteobacteria, reinforcing the ecological significance of QS due the importance of the CFB cluster in the marine media. These authors (Romero et al, 2008) also found a positive relationship between the presence of long-chain AHLs in the bacterial culture of *T. maritimum* strains and biofilm formation. In the last years, other fish pathogenic *Tenacibaculum* species have been isolated from eggs and larval stages (*T. ovolyticum*) of Atlantic halibut (*Hippoglossus hippoglossus* L.) (Hansen y col., 1992) and from juveniles and adult diseased turbot (*T. discolor, T. gallaicum*) and sole (*T. soleae*) (Piñeiro-Vidal et al., 2007, Piñeiro-Vidal et al., 2008 a, b). Some of these bacteria are usually isolated from fish (*T. discolor, T. soleae*) in mixed culture with *T. maritimum* and from seawater in fish-holding tanks (*T. gallaicum*) (Piñeiro-Vidal et al., 2007, 2008 a, b) and can be, therefore, their potential ecological competitors or simply organisms that use the QS signals produced by the other bacteria to initiate infection. Since the production of AHLs may represent a strong selective advantage upon other microorganisms that share the same habitat (Willians, 2007; Williams et al., 2007), profiling the AHL production of these species may provide a new insight in the establishment of infectious process in

tenacibaculosis. In the present work the “in vitro” and “in vivo” production of AHL’s activity by fish pathogenic *T. discolor* was examined in order to determine its ecological and pathological importance.

## Material and methods

### Bacterial strains

For the study of production of AHL-type QS signal molecules by *Tenacibaculum discolor*, the type strain of this species DSM 18842 was utilized. The strains of *Tenacibaculum* were routinely cultured on plates of *Flexibacter maritimus* medium (FMM) (Pazos et al., 1996) or Marine Agar (MA) at 25ºC for 24-48h. Stock cultures of the strains were stored at -70ºC in Microbank™ tubes (Prolab Diagnostics, Ontario, Canada).

### Extraction of AHLs produced "in vitro"

The influence of the culture medium in the production of AHLs by *T. discolor* DSM 18842 was tested in FMM broth and Marine Broth (MB, Difco). Culture of the strain DSM 18842 (20 ml) grown on the medium to be tested was inoculated in 1L Erlenmeyer flasks containing 300 ml of appropriate medium (FMM or MB) and incubated with shaking (100 rpm) for a period of 50 h at 25ºC. In order to evaluate the kinetics of AHLs production, at 0, 6, 24 and 50 hours of incubation, samples (50 ml) of the cultures were taken and the cells were removed by centrifugation (2000g x 5 min). The resultant supernatant was acidified to pH 2 with HCl 1M and maintained in a shaker at 200 rpm for 12 hours at 20ºC in order to avoid the disruption of the lactone ring of the AHLs (Yates et al., 2002). The AHLs were extracted twice with dichloromethane (1:1, v:v), the solvent was evaporated in a rotavapor at 40ºC and the final extract was suspended in 1 mL ethyl acetate. Extracts were conserved at -20ºC until use.

**HPLC-MS detection of AHLs**

AHLs were identified by using high performance liquid chromatography-mass spectrometry (HPLC-MS). The LC system used was an Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA). The column was a Zorbax Eclipse XDB-C18, 150 x 4.6 mm (5 µm particle size). The mobile phase was 0.1% formic acid in water (A), and methanol (B) and the flow rate was 0.4 ml/min. The gradient profile was as follows: 50% B over o to 10 min, a linear gradient from 50 to 90% B over 15 min, followed by 90% B for 25 min. The column was re-equilibrated for a total of 4 min. A 20µl volume of the sample in 0.1% formic acid in acetonitrile was injected onto the column. The MS experiments were conducted on an API4000 triple-quadrupole linear ion trap mass spectrometer (Applied Biosystem, Foster City, CA, USA) used in positive ion electrospray mode. Synthetic AHL with acyl side chain lengths of 4 to 14 carbons (Sigma) were used as standards. The AHLs were quantified by comparison with a calibration curve constructed for molecular ion abundance, using each of the appropriate AHL synthetic standards (Milton *et al*., 2001).

**Detection of AHL's in turbot tissues.**

For this assay healthy juvenile turbot (average weight of 15g) were maintained with a constant flow of seawater at 15°C with oxygen content above 8 mg/ L and salinity levels of 32%0. For pathogenicity assays, fish were anesthetized with MS222

(Sandoz) (100mg $ml^{-1}$) and infected by intraperitoneal inoculation of 0.1 ml of a bacterial suspension ($10^9$ cells per ml) in saline solution of the strain DSM 18842 (Piñeiro-Vidal et al., 2007). Mortalities were recorded daily for a 21 days period and were considered to be due to the inoculated strains if it was recovered from the internal organs of dead fish in pure culture. Samples of blood, liver and kidney of dead fish were inoculated on MA and FMM plates for recovery of the inoculated bacterium. For extraction of AHLs fish tissues were homogenized twice in acidified ethyl-acetate. After removing cell debris by filtration, the solvent was evaporated with nitrogen flux and the final extract was suspended in 200µl of acetonitril and analyzed by HPLC-MS as described above for the detection of AHLs produced *in vitro*.

**Effect of N-butyryl-DL-homoserine lactone (C4-HSL) on Oxidative burst of turbot macrophages**

For this assay healthy juvenile turbot (average weight of 15g and 75g) were acclimatised to laboratory conditions for at least 15 days in the conditions above described. For leucocyte collection, fish were anesthetized with MS222 and the head-kidney leucocytes were obtained aseptically and transferred to 5 ml of serum-free culture minimum essential medium (MEM-Earle, Biochrom, Cambridge, UK) supplemented with 100 IU $ml^{-1}$ penicillin, 100µg $ml^{-1}$ streptomycin (Biochrom), and 10 IU $ml^{-1}$ heparin (Sigma, USA). Cells suspensions were obtained by forcing the tissues through a nylon mesh (mesh size 100µm) in cold serum-free MEM. The resulting cell suspensions were placed onto Ficoll-Paque TM PLUS (Amersham

Biosciences, Sweden) and centrifuged at 400 x g for 20 min at 4ºC to remove erythrocytes and debris. The leucocyte rich interphase was collected with a Pasteur pipette, washed twice, counted and adjusted to $10^6$ cells/ml in MEM supplemented with antibiotics and 5% fetal calf serum (MEM-5) (Biochrom). Cell viability was determined by the trypan blue exclusion test. The oxidative burst was quantified using flow cytometry as a measure of intracellular reactive species using dihydrorhodamine 123 (DHR, 10 mM, stabilized in DMSO) (Sigma) as oxidative probe as previously described (El Morabit et al., 2006). For measurement of radical production, cells were treated with phorbol 12-myristate-13 acetate (PMA, 1mg $ml^{-1}$ in dimethyl sulfoxide) (Sigma) or with N-butyryl-DL-homoserine lactone (C4-HSL) (concentrations of 10µM, 1µM and 100nM in phosphate buffer, pH 5.0) (Sigma) or left untreated as controls. The level of intracellular fluorescence was measured in non-stimulated, PMA stimulated or C4-HSL treated cells. A total number of 0.5x $10^4$ events in the cell gate were analysed. The results are expressed as the difference in the geometric mean fluorescence intensity (GMFI) between samples with and without treatment. The assays were carried out in triplicate.

## Results and Discussion

### AHL detection and profiling of *T. discolor*

With the aim to detect and unequivocally identify the AHLs produced by the type strains of *T. discolor* DSM 18842 *in vitro*, the AHL profile in the extracts of acidified cell-free culture of *T. discolor* was examined by HPLC-MS. HPLC-chromatogram identified a peak with retention time similar to that of synthetic *N*-butyryl-L-homoserine lactone (C4-HSL) (Fig 1). The MS-spectrum of the peak unequivocally confirmed the identification of C4-HSL, being the only QS signal present in the acidified extracts of "in vitro" cultures of the strain DSM 18842 of *T. discolor* under the culture conditions tested (Fig 2). This profile is in deep contrast with the complex AHL profile reported for *T. maritimum* by Romero et al (2008). The presence of different AHL profiles in taxonomically close species and even within isolates of the same species has already been reported (Buch et al., 2003; Morohoshi et al., 2008). Divergences in the pool of AHLs produced even in closely related species reinforce the highly adaptative value of these molecules. The present findings add *T. discolor* to the growing group of fish pathogenic Gram-negative bacteria that uses AHLs and demonstrated the existence of different QS systems within members of the genus *Tenacibaculum*.

**Effect of growth conditions in the AHL profiles of *T. discolor***

In order to evaluate the existence of changes in the production of AHL associated to culture conditions and incubation time we have analyzed by HPLC-MS acidified extracts from bacterial cultures grown in MB and FMM during a period of 50h. No AHLs other than C4-HSL was found in any of the samples analyzed, which demonstrated that culture medium and period of incubation had not influence on the qualitative AHL profile of *T. discolor* strain DSM18842, at least under the conditions tested. These results contrast with those found in *T. maritimum* who presented a profile characterized by four AHL compounds during the first 24h of cultivation and only one major compound (C4-HSL) thereafter. On the contrary, HPLC-MS quantification analysis demonstrated differences in the amount and kinetic of C4-HSL produced by *T. discolor* in both media. When the strain was cultured in MB, no significative change in the concentration of C4-HSL was observed, remaining around a value of 60 ng/ml all over the period analyzed (50h) (Fig 3 A). No correlation was observed between AHL concentration and growth measured as absorbance (Fig 3 A and C). In contrast, the amount of C4-HSL increased with time in FMM culture, achieving a maximum after 24 hours and remaining constant thereafter (Fig 3 B). In this case, the stabilization of the C4-HSL production correlates with a change in the growth rate of the culture that did not achieve the stationary phase during the period analyzed (Fig 3 B and C). The existence of a great biological diversity in the mode and application of AHL production have previously been point out by Buchholtz et al. (2006).

**Detection of AHLs in infected turbot**

With the aim to investigate whether the AHL profile observed in "in vitro" cultures of the strain DSM 18842 were produced during infection of fish we carried out pathogenicity assays using turbot juveniles. The presence of C4-HSL in the acidified extracts of blood and fish tissues of healthy and experimentally infected fish was analyzed by HPLC-MS. C4-HSL was the ony AHL detected in the internal organs (kidney, spleen, and liver) of asymptomatic, moribund and dead infected fish (Table 1). C4-HSL activity was also detected in blood of asymptomatic and moribund but not in dead fish. The highest amount of C4-HSL compound was detected in spleen (ranging from 0.313 to 1.86 nmol/Kg fish) followed by liver (0.32 to 0.95 nmol/Kg fish) and kidney (0.061 to 0.517 nmol/Kg fish). Similar AHL levels have been detected in tissues of fish experimentally infected with *L. anguillarum* (Buch et al., 2003; Uchholtz et al, 2006). All the above indicates that the AHL-dependent quorum system of *T. discolor* DSM 18842 is functional "in vivo" and could play a role in colonization of fish tissues and in the pathogenesis of tenacibaculosis.

**Effect of C4-HSL on oxidative burst of turbot macrophages**

It has been described (Telford et al 1998; Smith et al., 2002) that some AHLs signal molecules may modulate the outcome of an infection by modulating the host immune response, functioning as virulence determinant per se. Respiratory burst activity (RBA) of phagocytes is essential for the elimination of invading microorganisms and constitutes one of the primary host defence mechanisms. The

"in vitro" studies performed by Telford et al. (1998) using human and murine leucocytes have demonstrated (that 3-oxo-C12 HSL modulates T-cell and macrophage functions, acting as a virulence determinant *per se*. To determine wheter C4-HSL may modulate the production of reactive compounds by turbot leucocytes, favouring the development of infectious processes by *T. discolor*, we have quantified by flow cytometry the RBA of head-kidney leucocytes treated with different C4-HSL concentrations (10µM, 1µM and 100nM). Neither increase nor reduction of intracellular fluorescence was observed in C4-HSL-treated leucocytes. Buch et al (2003) also reported that treatment of trout granulocytes with 3-oxo-C10-HSL from *L. anguillarum* do not affect this host defense mechanism.

In conclusion, *Tenacibaculum discolor* produces a single AHL, C4-HSL at detectable leves both "in vitro" and "in vivo"during experimental infection of turbot, which may play a role in the pathogenesis of the tenacibaculosis caused by this species. Further studies should be performed in order to clarify if QS circuits may play an ecological, physiological and/or pathological role within the Genus *Tenacibaculum*.

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## FIGURE LEGENDS

Fig. 1- HPLC-MS analysis of acidified extracts of the bacterial culture media of the strain of *Tenacibaculum discolor* DSM18842. A, AHLs standards. B, Sample of 6h. C. Sample of 24 h, D, sample of 48h.

Figure 2.- Mass spectrometry identification of C4-HSL. Mass spectrum of syntetic C4-HSL (A) and the correspondent peak (B) in the chromatograms of acidified extracts of the bacterial culture media of the strain of *Tenacibaculum discolor* DSM18842.

Figure 3.- Effect of the culture media and incubation time on the AHL production by the strain of *Tenacibaculum discolor* DSM18842 measured by HPLC-MS. The concentration of AHL compounds was evaluated using a standard curve constructed for molecular ion abundance using synthetic C4-HSL. A, Marine Broth; b, FMM broth and C, Growht curve of the strain growing in MB (---) and FMM broth (—).

**Acknowledgements**

This review was based in part on work supported by Grants, PGIDIT 04 RMA003E, PGIDIT 05 RMA 2350 1PR, INCITE 07PX2081174ES and PGIDIT 06 RMA 024E de la Xunta de Galicia.

Table 1.- Detection of C4-HSL in blood and internal organs of turbot experimentally infected with the strain DSM 18842 of *T. discolor*.

| **Turbot** | **Sample** | **HPLC-MS detection of C4-HSL (nmol/kg fish)** | **Recovery of the bacteria** |
|---|---|---|---|
| Non-infected (control) | Blood | 0.026 | - |
| | Spleen | 0.000 | - |
| | Kidney | 0.002 | - |
| | Liver | 0.025 | - |
| Asymptomatic | Blood | 0.486 | + |
| | Spleen | 1.144 | + |
| | Kidney | 0.517 | + |
| | Liver | 0.398 | + |
| Moribund | Blood | 0.018 | + |
| | Spleen | 0.313 | + |
| | Kidney | 0.061 | + |
| | Liver | 0.114 | + |
| Dead | Blood | 0.000 | - |
| | Spleen | 1.865 | + |
| | Kidney | 0.113 | + |
| | Liver | 1.430 | + |

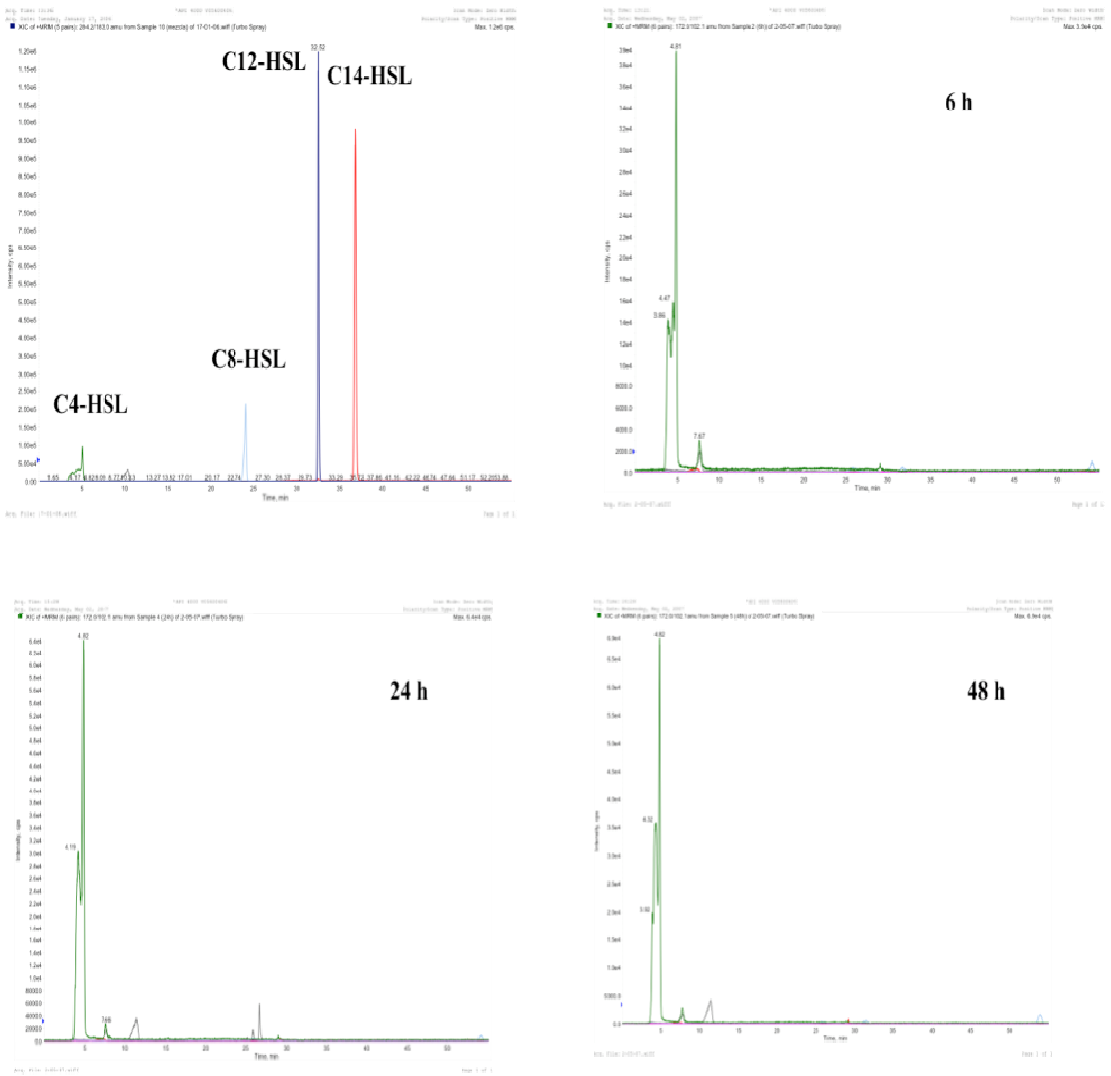



Fig. 1

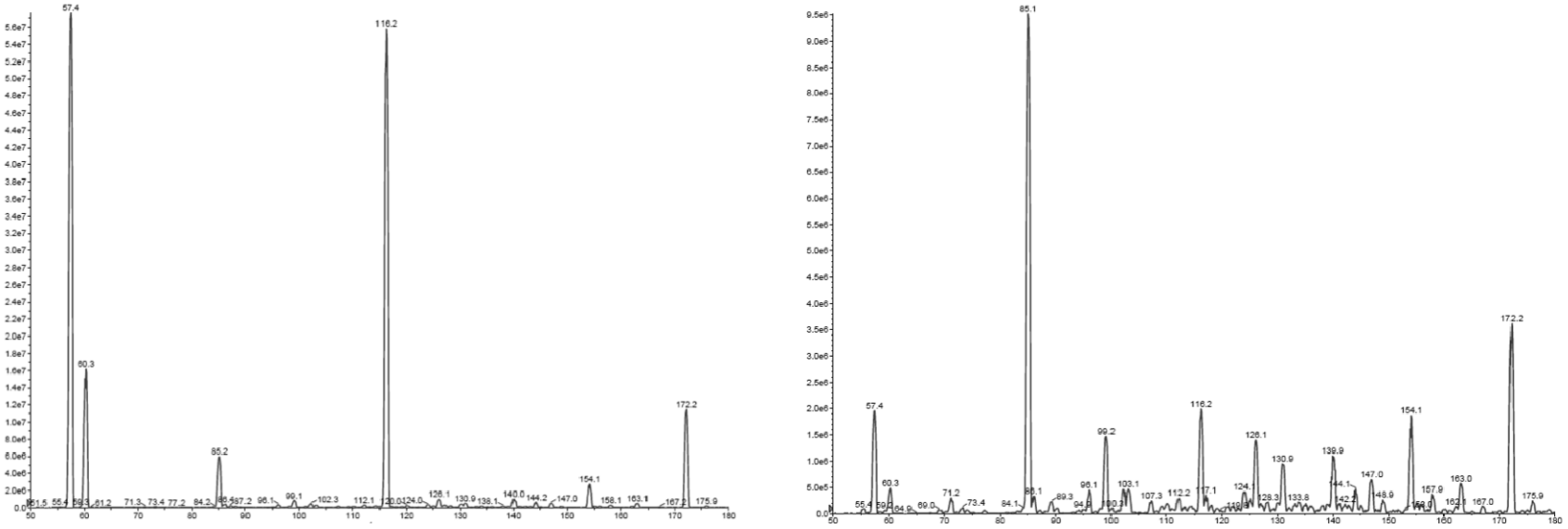
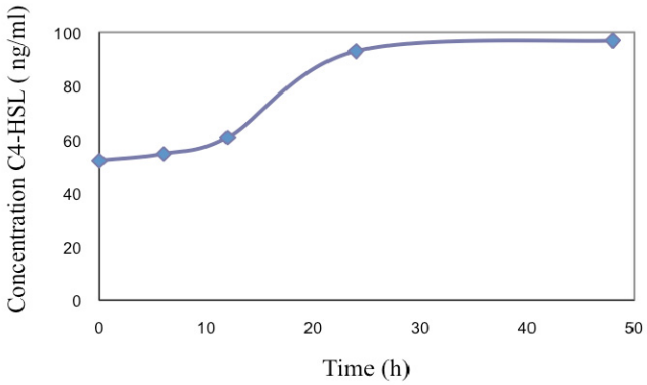
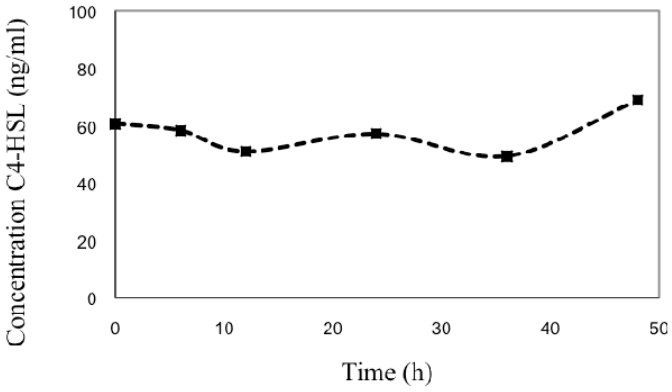

57.4
116.2
60.3
85.2
172.2
154.1
85.1
57.4
172.2
116.2
99.2
154.1
126.1
130.9
139.9
60.3
71.2
96.1
103.1
147.0
163.0

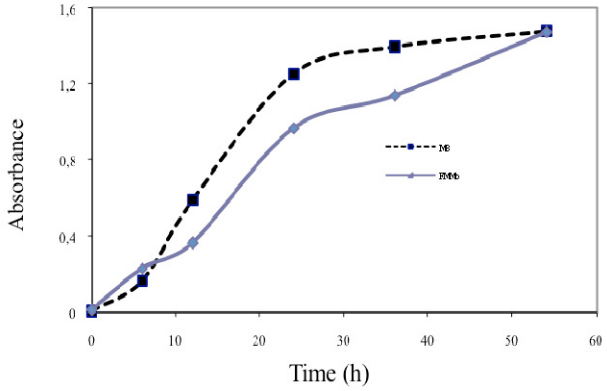
Fig. 2


Concentration C4-HSL ( ng/ml)
100
80
60
40
20
0
0
10
20
30
40
50
Time (h)

**A**


Concentration C4-HSL (ng/ml)
100
80
60
40
20
0
0
10
20
30
40
50
Time (h)

**B**


Absorbance
1,6
1,2
0,8
0,4
0
0
10
20
30
40
50
60
Time (h)

**C**

Fig. 3

# Capítulo VII. Discusión general

# VII.- Discusión general

La tenacibaculosis o flexibacteriosis marina, causada por *Tenacibaculum maritimum* es una enfermedad bacteriana responsable de importantes pérdidas económicas en granjas de cultivo de peces salmónidos y no salmónidos en todo el mundo. En Galicia esta enfermedad tiene una especial relevancia por ser una de las principales causas de mortalidad en los cultivos de rodaballo y lenguado. Los serotipos O1, O2, O3 y, en menor proporción, el serotipo O4 *de T. maritimum* se han aislado reiteradamente a partir de peces enfermos (Pazos, 1997; Santos y col., 1999; Santos, 2005). En los últimos años, en nuestro laboratorio se han aislado otras bacterias filamentosas a partir de peces juveniles y adultos con síntomas de tenacibaculosis así como del agua de los tanques de cultivo. Estos microorganismos han sido el objeto de estudio del trabajo de investigación reflejado en la presente memoria.

Con el fin de determinar el riesgo potencial que estos microrganismos podrían suponer para los cultivos de peces de relevancia económica para nuestra área, consideramos de interés realizar en primer lugar ensayos de patogenicidad. Los resultados obtenidos demostraron que los microorganismos aislados eran virulentos para rodaballo y lenguado cuando se inoculan por inyección intraperitoneal en dosis similares ($10^7$ UFC/pez) a las necesarias para producir mortalidad en el caso del conocido patógeno de peces *T. maritimum* (Magariños y col., 1995; Pazos, 1997). Además, hemos observado que tanto las cepas bacterianas filamentosas en estudio como las cepas de *T. maritimum* utilizadas como referencia en los ensayos de virulencia, carecen de especificidad de hospedador, siendo virulentas tanto para rodaballo como para lenguado. Por todo ello, sería necesario aplicar medidas que eviten la diseminación de estos microorganismos a otras especies susceptibles de padecer la enfermedad.

Un segundo aspecto abordado en la presente memoria fue el establecer la posición taxonómica de las bacterias filamentosas aisladas de sistemas de cultivo de rodaballo y lenguado. Para ello, las cepas bacterianas se caracterizaron empleando métodos microbiológicos convencionales, métodos de identificación basados en la reacción en cadena de la polimerasa (PCR) y métodos serológicos basados en la aglutinación. Los ensayos de caracterización fenotípica demostraron que estos microorganismos eran muy homogéneos en los caracteres morfológicos, fisiológicos y bioquímicos y muy similares a las cepas de *T. maritimum* empleadas como referencia en el estudio. Por tanto, el uso de sistemas de identificación convencionales no resultó ser de utilidad para el esclarecimiento

de la posición taxonómica de los aislados ni tampoco con fines epidemiológicos, al no permitir establecer diferencias entre cepas con un mismo origen geográfico u hospedador. La existencia de una gran homogeneidad a nivel de caracteres bioquímicos ha sido descrita previamente en la especie *T. maritimum*, principal agente causal de la tenacibaculosis en peces salvajes y cultivados (Pazos y col., 1993; Bernardet y col., 1994; Pazos, 1997; Santos y col., 1999; Avendaño-Herrera y col., 2004 a; Santos, 2005).

Dada la gran similaridad mostrada por los aislados a nivel bioquímico, consideramos de interés utilizar métodos moleculares y serológicos para su identificación. El uso de los protocolos de PCR y cebadores específicos (MAR 1 y MAR2) descritos por Bader y Shotts (1998) para la identificación de *T. maritimum*, permitieron demostrar que las bacterias filamentosas aisladas de sistemas de cultivo de rodaballo y lenguado no pertenecían a esta especie bacteriana. Del mismo modo, el ensayo de aglutinación en portaobjetos, empleando células bacterianas completas inactivadas con formaldehido y/o el antígeno "O" y antisuero frente a los cuatro serotipos descritos en la especie *T. maritimum* por Pazos (1997), demostraron que las cepas en estudio no están relacionadas serológicamente con esta especie bacteriana. Además, el ensayo de aglutinación en portaobjetos utilizando sueros específicos frente a algunas de las bacterias filamentosas en estudio puso de manifiesto la existencia de variabilidad serológica entre los aislados, pudiéndose distinguir cuatro grupos serológicos. La existencia de variabilidad en las características serológicas han sido descritas en otras bacterias aisladas a partir de sistemas de cultivo de peces como *T. maritimum*, *Listonella anguillarum; L. pelagia* (*Vibrio pelagius*) y *V. splendidus* (Sørensen y Larsen, 1986; Austin y col, 1995; Santos y col., 1995; Santos y col., 1996; 1997; Pazos, 1997; Santos y col., 1999; Avendaño-Herrera y col., 2004 a).

En un reciente trabajo, Bernardet y col. (2002) han propuesto unos caracteres estándares mínimos para el establecimiento de una especie dentro de la familia *Flavobacteriaceae*. Entre los criterios descritos para la determinación de si un microorganismo representa una nueva especie se recomiendan: i) la amplificación de la secuencia del gen ARNr 16S, ii) la determinación del contenido en bases pirimidínicas en el contenido total del ADN, iii) las pruebas de hibridación de ADN-ADN y iv) el estudio de la composición de ácidos grasos.

Debido a la falta de resultados concluyentes derivados del análisis de caracteres fisiológicos y bioquímicos observadas en nuestro estudio, se optó en primer lugar por la secuenciación del gen ARNr 16S y la realización de un análisis filogenético para definir la

posición taxonómica de nuestros aislados. En primer lugar, se analizaron las secuencias del gen 16S ARNr obtenidas para las cepas LL04.11.1.1 (1512 pb) y LL04 12.1.7 (1506 pb) (aisladas de lenguado) y para la cepa A37.1 (1518 pb) (aislada del agua del tanque de cultivo de rodaballos afectados de tenacibaculosis) y se compararon con las secuencias de todas las especies de *Tenacibaculum* descritas hasta el momento y con las de otros miembros de la Familia *Flavobacteriaceae* disponibles en las bases de datos GenBank/EMBL. Los resultados obtenidos indicaron que las cepas LL04 12.1.7, LL04 11.1.1 y A37.1 forman un grupo robusto con las otras especies del Género *Tenacibaculum*. Además, se observó que las cepas LL04.11.1.1 y A37.1 estaban relacionadas estrechamente entre sí (98,4% de similaridad) y en menor grado con la cepa LL04 12.1.7 (similaridad del orden del 96%). Las cepas LL04 11.1.1, A37.1 y LL04 12.1.7 mostraron un grado de similaridad con la cepa tipo de las especie *T. maritimum* (94,2%, 94,3% y 94,8%, respectivamente), inferior al valor considerado como mínimo (97%) para la inclusión de dos taxones en la misma especie en base al análisis de similaridad de la secuencia del gen ARNr 16S (Stackebrandt y Goebel, 1994; Stackebrandt y Ebers, 2006). Estos resultados indican claramente la no pertenencia de las bacterias filamentosas en estudio a la especie *T. maritimum*, corroborando los resultados obtenidos con la técnica de PCR y ensayo de aglutinación en portaobjetos descritos anteriormente. Teniendo en cuenta este mismo criterio se concluyó que la cepa LL04 12.1.7, que mostró un porcentaje de similaridad de secuencia del gen ARNr 16S inferior al 97% con las cepas tipo de todas las especies de *Tenacibaculum* descritas hasta el momento, representa una nueva especie dentro de éste Género. En cambio las cepas LL04 11.1.1 y A37.1 mostraron una gran similaridad con la cepa tipo de la especie *T.litoreum* (99,4% y 98,4% de similaridad, respectivamente). En un reciente trabajo de revisión, Stackebrandt y Ebers (2006) recomiendan que los ensayos de reasociación DNA-DNA se utilicen sólo cuando el análisis de la secuencia del gen ARNr 16S revele una similaridad del orden del 98,7-99%. Teniendo en cuenta lo expuesto, se realizaron análisis de hibridación DNA-DNA entre las cepas A37.1 y LL04 11.1.1 y entre éstas y la cepa tipo de *T. litoreum* y se procedió a determinar el contenido en G+C de las tres cepas en estudio (A37.1, LL04 11.1.1 y LL0412.1.7). Los ensayos de hibridación de DNA mostraron sólo un 42,8% de homología entre las cepas LL04 11.1.1 y A37.1 y una homología de tan sólo el 39,6% y del 40,2%, respectivamente con la cepa tipo de *T. litoreum*. Estos valores están muy por debajo del 70% de homología, valor considerado como mínimo para asignar dos taxones a la misma especie (Stackebrandt y Goebel, 1994). Con respecto al contendido en G+C de las cepas LL04.11.1.1 (32,1%) y A37.1 (32,7%) está en el rango de los valores (30,0-35,2%) descritos previamente (Suzuki y col., 2001)

para el Género *Tenacibaculum*. Sin embargo, el contenido en G+C de la cepa LL04 12.1.7 fue de 29,8%mol, siendo el valor más bajo descrito hasta el momento para las especies del Género *Tenacibaculum*.

En conclusión los datos fenotípicos y genéticos obtenidos a lo largo de esta investigación indican que las cepas en estudio representan tres nuevas especies dentro del género *Tenacibaculum* para las cuales se han propuesto los nombres específicos de *Tenacibaculum discolor* sp. nov., *T. gallaicum* sp. nov. y *Tenacibaculum soleae* sp. nov.

El estudio de los perfiles de ácidos grasos tiene un gran valor a nivel taxonómico, permitiendo la diferenciación de un nuevo taxón con respecto a otros ya existentes en el mismo género y también entre aislados de un mismo taxón. Además, el análisis cualitativo y cuantitativo de los ácidos grasos permite detectar adaptaciones de las bacterias a la composición lipídica de sus hospedadores o micronichos (Bøe y Gjerde, 1980; Lambert y col., 1983; Komagata y Suzuki, 1987; Welch, 1991; Kämpfer y col., 1994; Toranzo y col., 1994; Romalde y col., 1995; Vandamme y col., 1996; Steele y col., 1997; Dijkshoorn y Towner, 2001; Bernardet y col., 2002; Inglis y col., 2003; Shoemaker y col., 2005). Por todo ello, el análisis del perfil de ácidos grasos puede tener un importante papel a nivel de estudios taxonómicos y epidemiológicos. Sin embargo, existen pocos datos referentes a la composición de ácidos grasos de las especies del Género *Tenacibaculum* (Pazos, 1997; Suzuki y col., 2001) y pocos estudios en los que se haya utilizado los perfiles de ácidos grasos con fines epidemiológicos, incluyendo patógenos de peces (Shoemaker y col, 2005).

Por todo ello, en el presente estudio nos propusimos determinar la utilidad del análisis de los perfiles de ácidos grasos en la diferenciación taxonómica y epidemiológica de bacterias del Género *Tenacibaculum* patógenas para peces. En general, los perfiles de ácidos grasos de las cepas analizadas son similares a los descritos previamente para el género *Tenacibaculum* (Bowman y col., 1998; Jung y col., 2006; Sheu y col., 2007) aunque se observaron ciertas variaciones en cuanto al contenido en determinados ácidos grasos que pueden ser debidas a las diferentes condiciones de cultivo, la propia cepa o el procedimiento analítico utilizado. Por otra parte, se observó que los perfiles de ácidos grasos de las cepas de *T. maritimum*, *T. discolor*, *T. gallaicum*, no diferirían entre sí de forma significativa excepto por unos pocos ácidos grasos (iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH, iso-$C_{15:1}$ G, iso-$C_{16:0}$ y $C_{17:1}$ ω6c, y C16:1 ω7c y/o iso-$C_{15:0}$ 2OH). Sin embargo, los perfiles de ácidos grasos de *T. ovolyticum* y *T. soleae*, mostraban diferencias cualitativas y cuantitativas importantes con respecto a los observados en las otras especies de *Tenacibaculum* patógenas para peces. El análisis de componentes principales demostró que

*T. ovolyticum* y *T. soleae* se separan del resto de especies patógenas de peces en base a cuatro (iso-$C_{14:0}$, $C_{16:0}$ 3OH, anteiso-$C_{15:0}$ e iso-$C_{13:0}$) y tres ácidos grasos ($C_{15:1}$ ω6c, $C_{15:0}$, $C_{15:0}$ 3OH), respectivamente, lo cual puede ser utilizado como criterio taxonómico. Además, las cepas de *T. maritimum* se separaron en dos grupos asociados con el hospedador/origen geográfico en base a dos ácidos grasos iso-$C_{15:1}$ G e iso-$C_{15:0}$ 3OH, los cuales pueden ser utilizados como marcadores para estudios epidemiológicos. En contraste, *T. discolor* y *T. gallaicum* están estrechamente relacionadas en base a los perfiles de ácidos grasos, lo cual concuerda con los resultados obtenidos mediante el análisis filogenético realizado en el presente estudio. En conclusón, el análisis de ácidos grasos tiene un indudable valor en la caracterización taxonómica de especies patógenas de peces del género *Tenacibaculum* y además permite la diferenciación de las cepas de *T. maritimum* en función de su origen (especie de pez/ área geográfica).

El análisis de los componentes de la envoltura celular (LPS y proteínas) es una de las características mínimas que han de ser evaluadas para la descripción de nuevas especies dentro de los géneros incluidos en la Familia *Flavobacteriaceae* (Bernardet y col., 2002), incrementan la eficacia de las técnicas serológicas de identificación basadas en la aglutinación y permiten establecer las relaciones inmunológicas entre los antígenos de diferentes cepas, proporcionando información de interés para la formulación de vacunas eficaces. El análisis de las proteínas de membrana reveló la presencia de bandas con movilidad electroforética similar en las cepas de *T. maritimum*, *T. soleae*, *T. discolor* y *T. gallaicum*. La existencia de homogeneidad en los perfiles electroforéticos de proteínas de la envoltura celular han sido descritos previamente en *T. maritimum* (Pazos y col., 1993; Bernardet y col, 1994; Pazos, 1997; Avendaño-Herrera y col, 2004 a). Además, las cepas de *T. discolor*, *T. gallaicum* y *T. soleae* mostraban un perfil de LPS caracterizado por la presencia de dos fracciones de alto y bajo peso molecular, en contraste con el perfil "en escalera" observado en los aislados de *T. maritimum* en el presente trabajo y en estudios previos (Pazos y col., 1993; Pazos, 1997; Avendaño-Herrera y col., 2004 a). Las diferencias en los perfiles electroforéticos de los LPS de la envoltura celular observados en este estudio podrían ser utilizados como método para la diferenciación de *T. maritimum* de otras especies de *Tenacibaculum* patógenas de peces.

Los ensayos de *immunoblot* utilizando anti-sueros obtenidos en conejo han demostrado que las proteínas y LPS de las especies *T. discolor*, *T. gallaicum* y *T. soleae* no están relacionados inmunológicamente entre sí y tampoco con los componentes de la envoltura celular de *T. maritimum*. En otras bacterias patógenas de peces como *L.*

*anguillarum*, *Aeromonas* móviles y *T. maritimum* (Santos y col., 1995; Santos y col, 1996; Pazos, 1997) se ha demostrado que los productos extracelulares (ECP) juegan un papel en la virulencia, son inmunogénicos para peces y presentan LPS y proteínas inmunológicamente distintos de los componentes de la envoltura celular bacteriana. En el presente estudió se analizaron los componentes celulares y extracelulares de *T. discolor*, *T. gallaicum* y *T. soleae* mediante *immunoblot* usando los antisueros obtenidos frente a las células completas de las cepas tipo de cada especie, observando que los antígenos celulares y extracelulares de dichas especies están relacionados inmunológicamente. Además, cuando se utilizó suero de rodaballos hiperinmunizados con la cepa LPV1.7 de *T. maritimum*, utilizada para elaborar la vacuna anti-tenacibaculosis comercializada actualmente para el control de la tenacibaculosis, sólo se observó reacción con los LPS y proteínas homólogas y no con los de las cepas de las otras especies patógenas para peces del género *Tenacibaculum*. Todos estos resultados sugieren que la inclusión de las variantes antigénicas de las especies *T. discolor*, *T. gallaicum* y *T. soleae*, predominantes en un área dada, en la formulación de vacunas frente a la tenacibaculosis podría incrementar su eficacia.

En los últimos años se ha descrito la producción de señales de comunicación celulares mediadas por N-acil –L-homoserín lactona (AHLs) en una gran variedad de bacterias Gram-negativas. Estas moléculas están implicadas en un mecanismo de regulación de la expresión de genes dependiente de la densidad celular conocido como "quorum sensing" (QS) (Williams y col, 2007). Las moléculas señal o autoinductoras juegan un importante papel en la expresión de factores de virulencia necesarios para la colonización de la piel y tejidos internos (formación de biofilms, producción de proteasas, sideróforos, toxinas…) en una gran variedad de bacterias patógenas para el hombre, animales homeotermos y peces (Manefield y col., 2000; Buch y col., 2003; Bruhn y col., 2005; Buchholtz y col., 2006; Kastbjerg y col., 2006; Williams y col., 2007). Recientemente, Romero y col (2008) han descrito la presencia de AHLs (C4-HSL, C8-HSL, C12-HSL y C4-HSL) en el sobrenadante de los cultivos de cepas de *T. maritimum* y su posible relación con la formación de biofilms. Dado que la especie *T. discolor* es aislada con frecuencia en cultivo mixto con *T. maritimum* a partir de peces afectados de tenacibaculosis, consideramos de interés estudiar la producción "in vitro" e "in vivo" de AHLs por *T. discolor* con el fin de valorar su posible papel en la supervivencia de este microorganismo y en su patogenicidad. El análisis mediante cromatografía-espectrometría de masas demostró que la cepa tipo de la especie *T. discolor* DSM 18842 produce N-butiril-L-homoserín lactona como única señal de QS en las condiciones de cultivo probadas

(medios FMM o MB, a 25º C con agitación de 100 rpm y período de incubación de 50h). Este resultado contrasta con el complejo perfil de AHLs descrito en *T. maritimum* por Romero y col (2008). La producción de diferentes AHLs por especies relacionadas taxonómicamente e incluso por diferentes cepas de una misma especie ha sido descrito con anterioridad en el patógeno de peces *Listonella anguillarum* (Buch y col., 2003) y en *Chromobacterium violaceum* (Morohoshi y col., 2008). Otro aspecto de interés del presente estudio ha sido la detección de la producción de C4-HSL por la especie *T. discolor*, como única señal de QS, durante la infección experimental de rodaballos. Estos resultados indican que el sistema de QS dependiente de AHL es funcional "in vivo" y puede desempeñar un papel en la colonización de los tejidos del pez y en la patogénesis de la tenacibaculosis. Además, con el fin de evaluar si la C4-HSL puede tener un papel inmunomodulador, regulando la respuesta inmune del rodaballo en beneficio del patógeno, hemos estudiado si el tratamiento con la lactona tiene algún efecto sobre la actividad respiratoria de los leucocitos del riñón anterior de rodaballo. No se ha detectado efecto inmunosupresor ni inmunoestimulador sobre los leucocitos de rodaballo en los ensayos "in vitro", sugiriendo que la C4-HSL de *T. discolor* no actúa como un factor de virulencia "per se". Buch y col (2003) tampoco observaron efecto alguno sobre la actividad respiratoria de los leucocitos de trucha tras el tratamiento con la lactona 3-oxo-C10-HSL de *L. anguillarum*. Es necesario realizar más estudios con el fin de esclarecer si los sistemas de QS pueden desempeñar un papel ecológico, fisiológico y/o patológico dentro del Género *Tenacibaculum*.

# Capítulo VIII. Conclusiones

# VIII.- Conclusiones

En base a los resultados obtenidos podemos concluir que:

- Las cepas de bacterias filamentosas aisladas de sistemas de cultivo de rodaballo y lenguado presentan una gran similaridad a nivel bioquímico con la especie *Tenacibaculum maritimum* pero difieren de esta especie bacteriana a nivel genético y serológico.

- Las cepas de bacterias filamentosas en estudio son virulentas para rodaballo y lenguado, produciendo mortalidades entre el 85-100% cuando los peces se infectan por inyección intraperitoneal con dosis de $10^7$ UFC/pez.

- El uso de los protocolos de PCR descritos para la identificación de *T. maritimum* y la técnica serológica de aglutinación en portaobjetos utilizando anticuerpos policlonales y células inactivadas con formaldehido son de utilidad para el diagnóstico de la tenacibaculosis, permitiendo diferenciar *T. maritimum* de otras bacterias filamentosas patógenas para el rodaballo y lenguado.

- El análisis de la secuencia completa del gen que codifica el RNAr 16S, la determinación del contenido en G+C y los ensayos de hibridación DNA-DNA indican que las bacterias filamentosas aisladas de los sistemas de cultivo de rodaballo y lenguado representan tres nuevas especies dentro del género *Tenacibaculum* para las cuales se ha propuesto el nombre específico de *Tenacibacullum gallaicum*, *Tenacibaculum discolor* y *Tenacibaculum soleae.*

- El análisis del perfil de ácidos grasos es una herramienta útil en estudios taxonómicos y epidemiológicos de bacterias pertenecientes al género

*Tenacibaculum*, aisladas de sistemas de cultivo marinos, ya que permite diferenciar *T. maritimum* de otras bacterias del género *Tenacibaclum* patógenas de peces y diferenciar las cepas de *T. maritimum* en función de su orígen.

- Las especies *T. discolor*, *T. gallaicum* and *T. soleae* presentan proteínas y lipopolisacáridos de la envoltura celular diferentes inmunológicamente.

- Las proteínas y lipopolisacáridos de la envoltura celular y de los productos extracelulares de las especies *T. discolor*, *T. gallaicum* and *T. soleae* están relacionados a nivel inmunológico.

- La especie *Tenacibaculum discolor* produce una única señal de quorum, *N*-butiril-L-homoserín lactona (C4-HSL), a niveles detectables “in vitro” e “in vivo”, lo cual sugiere que puede tener un papel en la patogénesis de la tenacibaculosis.

- El tratamiento con C4-HSL de los leucocitos del riñón anterior del rodaballo no altera su actividad respiratoria, lo cual indica que esta señal de quorum no actúa como un factor de virulencia *per se*, modulando este mecanismo de defensa primario del rodaballo.

# Capítulo IX. Bibliografía

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