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

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

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

Extraction of lipopolysaccharides (LPS) and electrophoretic analysis. The extraction of LPS present in the cell envelope of the isolates was performed using the methods of Hitchcock & Brown (1983). Bacteria

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

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

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

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

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

RESULTS AND DISCUSSION

Biochemical characterization

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

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

Serological characterization

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

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

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

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

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

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

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

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

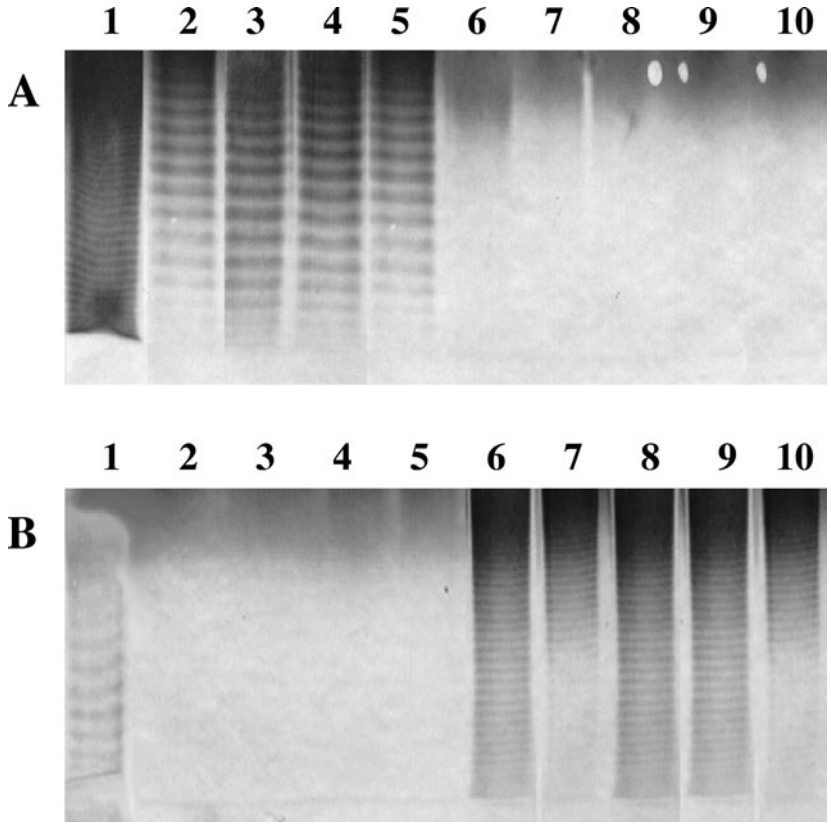

Fig. 1. *Tenacibaculum maritimum.* Western blot analysis of lipopolysaccharides of strains, using antisera raised against (A) sole strain PC503.1 and (B) turbot strain PC424.1 Lane 1: NCIMB 2153; 2: PC477.1; 3: PC503.1; 4: AZ203.1; 5: PC560.1; 6: PC424.1; 7: PC394.1; 8: RI93.1; 9: RM256.1; 10: LPV1.7.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

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

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

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

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

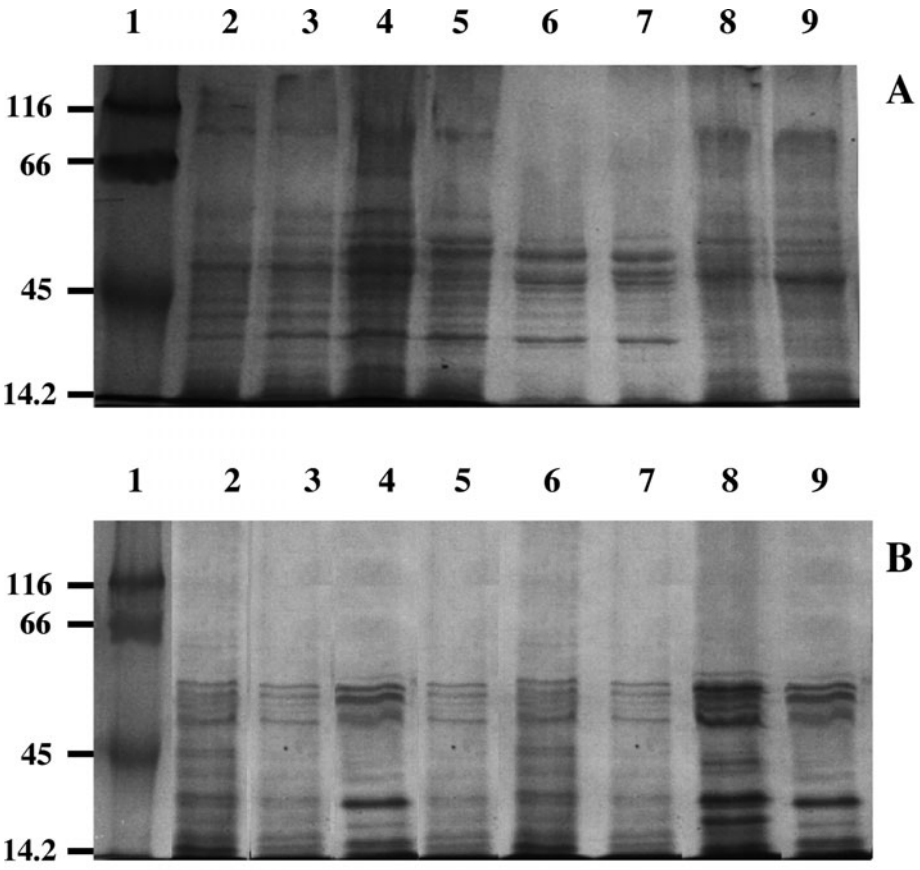

Fig. 2. SDS-PAGE of (A) total and (B) outer membrane proteins of *Tenacibaculum maritimum* strains. Lane: 1: molecular size markers; 2: PC424.1; 3: RM256.1; 4: NCIMB 2153; 5: NCIMB 2158; 6: LR2P; 7: PC528.1; 8: PC503.1; 9: PC560.1. Numbers on left: positions of molecular size marker (kDa)

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