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Identification of *Tenacibaculum maritimum* strains from marine farmed fish in Greece

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Tenacibaculum maritimum (formerly *Cytophaga marina* and *Flexibacter marinus*) is an opportunistic bacterial pathogen that causes a disease called 'flexibacteriosis' in marine fish. Other names that have been used for flexibacteriosis are 'gliding bacterial disease', 'eroded mouth syndrome' and 'black patch necrosis'. The disease is mainly characterised by haemorrhagic skin lesions and this condition is also associated with fin and gill disease. In a few cases, systemic forms have also been reported. Initially, *T maritimum* was isolated from marine fish cultured in Japan in 1979 (Hikida and others 1979). The first report in Europe was published in 1982, in Dover sole (*Solea solea*) (Campbell and Buswell 1982). Flexibacteriosis is currently widely spread in Europe, the USA and Japan, affecting many marine fish species (Vatsos 2007) Based on phenotypic and biochemical characteristics, *T maritimum* is generally considered as a homogeneous taxon. Methods for isolation and identification of the pathogen include the following: observation of accumulations of long rods in wet mounts or gram-stained preparations obtained from gills or skin lesions; isolation in various culture media, especially Anacker and Ordal, Marine Agar, *Flexibacter maritimus* medium (FMM; Pazos and others 1996); and PCR (Toyama and others 1996, Avendaρo-Herrera and others 2004). Greece is the main producer of sea bream (*Sparus aurata*) and sea bass (*Dicentrachus labrax*) in the Mediterranean area. Flexibacteriosis is considered as one of the main bacterial diseases in these two species, especially when stressful conditions occur (Toranzo and others 2005). In the present study, several strains of *T maritimum* were collected from various marine farming sites in Greece, in order to investigate whether the strains present in Greece share a similar biochemical profile. This information is currently not available and is critical in many epidemiological studies. Eleven strains of *T maritimum* were collected from many areas of Greece (Table 1). These strains were isolated from various field samples, which were sent to the Ichthyology Laboratory, School of Veterinary Medicine, University of Thessaly, between March and June 2010.

These samples originated from different fish farms, in which outbreaks of flexibacteriosis occurred, and were either swabs collected from skin lesions or dead fish, showing lesions of the disease, on ice. In both cases, cultures on five different media were prepared in order to examine which medium was more appropriate for the initial recovery. The media were as follows: Marine Agar (MA), Marine Agar + 2 mg/l flumequine (MA-F), FMM, FMM + 2 mg/l flumequine (FMM-F) and FMM + 4 g/l neomycin (FMM-N) (Table 1). The media were prepared according to previously described methods (Pazos and other 1996, Avendaρo-Herrera and others 2005). As previous studies (Pazos and other 1996, Avendaρo-Herrera and others 2008) have indicated that *T maritimum* is generally resistant to flumequine and neomycin, in this study, the potential of using these two antibiotics in the culture media in order to inhibit the growth of competitive sensitive bacterial species was also tested. All cultures were incubated at 19°C for seven days. This temperature was chosen as this was similar to the water temperature at the farming sites from which the samples were originated. After the initial propagation, individual colonies resembling typical *T maritimum* colonies were collected and recultured on fresh media, using the same culture conditions as before. Bacterial samples from the second cultures were then collected and the confirmation of the bacterial species was performed using the phenotypic characteristic of the bacteria as well as the PCR method suggested by Toyama and others (1996) for the detection of *T maritimum*. Once the confirmation was completed, each strain was given an identification number, which consisted of three parts: an initial number according to the geographical area (1 North Ionian Sea, 2 South Ionian Sea, 3 South Aegean Sea, 4 North Aegean Sea), the exact farming site, and the fish species (LV Sea bass, TS Sea bream) (Table 1). In order to examine the biochemical profiles of the isolated and identified strains, four commercial miniaturised systems were used: API 20E, API 20NE, API 50CE and API ZYM. The methods were carried out according to the manufacturer's guidelines (bioMırieux). Almost all strains grew better on FMM-F medium (Table 1), although two of them appeared sensitive to flumequine and grew only on MA. The colonies on FMM were round with uneven edges and yellowish (Fig 1a). The colonies on MA were round, light yellow and semitransparent (Fig 1b). Concerning the biochemical profiles, all the isolated strains exhibited identical profiles using the API 20E and the API 20NE systems. The API 20E numerical profile of all strains was 040100410, while the API 20NE profile was 1010004. Concerning the API ZYM system, positive results were noted only in the first 11 reactions

for all strains (Table 2), while no reactions were observed in all carbohydrate substrates in the API 50CE.

TABLE 1: Growth of *Tenacibaculum maritimum* strains on five culture media

	Culture medium				
Strain	MA	MA-F	FMM	FMM-F	FMM-N
1.SGK23.LV	-	-	X	Y	-
1.SGK4.LV	-	-	X	Y	-
1.PROPA9.TS	-	-	X	Y	-
1.PROPB4.LV	-	-	X	Y	Y
2.XL1.LV	Y	-	-	-	-
2.XL2.LV	Y	-	-	-	-
4.SFK2.LV	-	-	-	Y	-
4.SFK3.LV	-	-	-	Y	Y
4.SFK4.LV	-	-	X	Y	-
3.KAN3.LV	-	-	-	Y	-
3.KAN4.LV	-	-	X	Y	-

-No growth, X Colonies of *T maritimum* mixed with colonies of other bacterial species, Y Clear colonies of *T maritimum*
MA Marine Agar, MA-F MA + 2 mg/l flumequine, FMM *Flexibacter maritimum* medium, FMM-F FMM + 2 mg/l flumequine, FMM-N FMM + 4 g/l neomycin

TABLE 2: Biochemical profiles of the 11 *Tenacibaculum maritimum* strains using API ZYM (bioMérieux). The table shows the results of first 11 reactions

Strain	CON	ALK	C_4	C_8	C_{14}	ARYL	VAL	CYS	TRY	CHR	ACP	NP
1.SGK23	-	5	3	4	2	5	5	3	1	1	5	5
1.SGK4	-	4	2	4	2	5	5	4	-	2	5	5
1.PROPA9	-	5	3	4	1	5	5	4	1	2	5	5
1.PROPB4	-	5	3	4	2	5	5	3	1	1	5	5
2.XL1.LV	-	5	3	3	2	5	5	4	1	1	5	5
2.XL2.LV	-	5	3	4	2	5	5	4	1	1	5	5
4.SFK1	-	5	3	4	2	5	5	4	1	2	5	5
4.SFK2	-	5	2	4	1	5	5	4	1	1	5	5
4.SFK3	-	5	2	4	1–2	5	5	4	-	2	5	5
3.KAN3	-	5	3	3	2	5	5	3	1	1	5	5
3.KAN4	-	5	3	4	2	5	5	4	1	1	5	5

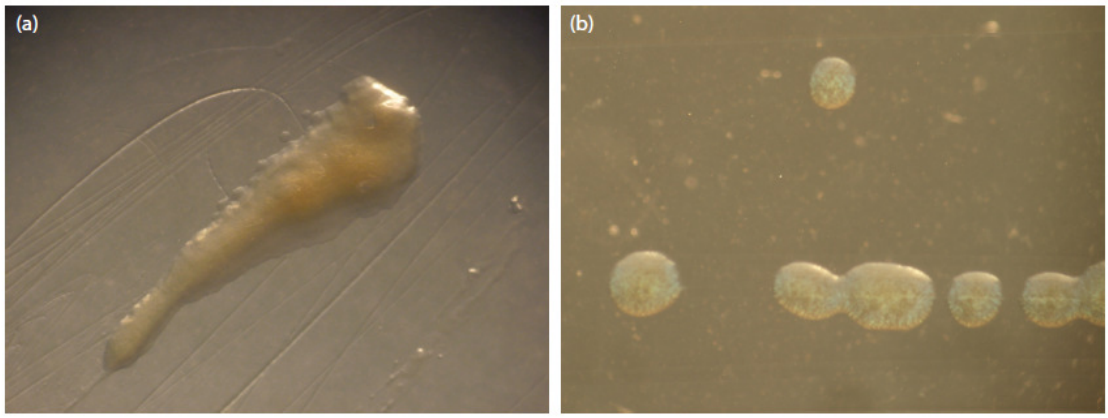

FIG 1: Colonies of *Tenacibaculum maritimum* on (a) FMM and (b) MA agar

T maritimum is a very common bacterium of the marine aquatic environment (Salati and others 2005), in which it can survive for months outside the fish, especially when it is protected within organic material, such as mud or fish mucus (Avendapo-Herrera and others 2006). The bacterium grows slowly on all the culture media examined so far and in many cases, its growth is inhibited by other fast-growing bacterial species. Among the five culture media that were used in this study, FMM and FMM-F provided the best recovery. These results are in agreement with those of earlier studies (Pazos and other 1996, Avendapo-Herrera and others 2008). Two strains appeared to be sensitive to flumequine. Avendapo-Herrera and others (2008) conducting a survey on some marine fish farms in north-west Spain and Portugal showed that depending on the farm, 25 to 60 per cent of the *T maritimum* strains examined appeared resistant to this antibiotic. Thus, it suggests that the inhibitory medium FMM-F can not be used alone for the initial recovery, as it can exclude some sensitive strains, although it provides clear colonies of *T maritimum*. The miniaturised API systems are commonly used to differentiate several

bacterial species. The biochemical profiles of the strains used appeared almost identical and minor differences were observed when API ZYM was used. These profiles are similar to those previously reported (Buller 2004) confirming the biochemical homogeneity of this species. Studies using serological and molecular biology methods, identified three groups, according to the host fish species (Vatsos 2007). In conclusion, this study has confirmed that the T *maritimum* strains that exist in farmed sea bass and sea bream in Greece exhibit almost identical biochemical profiles, similar to those reported from other geographical areas. In addition, FMM appears to be the most appropriate culture medium for the initial recovery from field samples.

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