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ORIGINAL ARTICLE

Diversity of *Shewanella* population in fish *Sparus aurata* harvested in the Aegean Sea

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Abstract

Aims: To study the diversity of *Shewanella* population in *Sparus aurata* fish harvested in the Aegean Sea, as well as to elucidate the influence of fish storage conditions on the selection in *Shewanella* strains.

Methods and Results: A total of 108 strains of *Shewanella* spp. were isolated from *Sparus aurata* during storage under various conditions. Conventional phenotypic analysis along with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins and 16S rRNA sequence analysis were used for the characterization of the strains. Numerical analysis of whole cell protein profiles showed that the isolates were separated into two distinct clusters A and B with 47% similarity. Cluster B was further subdivided into two subclusters B1 and B2 with 70% similarity. One strain could not be assigned to any of these groups. The different ability of isolates to utilize deoxycholate, D-saccharate, D-glucuronate, N-acetyl-glycosamine, D-maltose, gluconate and citrate, as well as the different type of metabolism on the Hugh and Leifson medium distinguished the different *Shewanella* biogroups, as these were defined by the SDS-PAGE analysis. Representative strains from the three biogroups were further investigated by 16S rRNA sequence analysis and showed more than 99·4% similarity.

Conclusions: Significant similarities between the isolates and the type strains of *S. baltica*, *S. putrefaciens* and *S. oneidensis* at both phenotypic and molecular level signalize that the new isolates are closely related with the above *Shewanella* species, but do not provide a clear evidence to which of these species they belong.

Significance and Impact of the Study: The lack of information about the diversity of *Shewanella* population in *Sparus aurata* fish originated from Mediterranean Sea could be confronted using conventional phenotypic techniques, SDS-PAGE analysis of whole cell proteins and 16S rRNA sequencing.

Introduction

Autolysis of muscle cells, chemical oxidation of lipids, and bacterial growth and metabolism are among the causes for fish spoilage. There is no doubt, however, that microbiological activity is by far the most important factor influencing fish quality (Gram and Huss 1996). Indeed, *Shewanella putrefaciens*, *Pseudomonas* spp., *Photobacterium phosphoreum*, *Brochothrix thermosphacta*, *Aeromonas* spp. and lactic acid bacteria are not only members of the microbial association found in fish from temperate waters (Koutsoumanis and Nychas 1999; Dalgaard 2000), but they also contribute significantly to the spoilage of fish stored under different conditions. However, not all

these bacteria contribute at the same extend to fish spoilage. For example, *S. putrefaciens* is the specific spoilage organism (SSO) of marine cold-water fish stored in ice, whereas *Ph. phosphoreum* is the SSO of fish stored under modified atmosphere conditions (Gram and Dalgaard 2002). On the contrary, *Pseudomonas* spp. and *Shewanella* spp. were found to be the SSO in fish from Mediterranean Sea (temperate waters) stored aerobically in ice (Koutsoumanis and Nychas 1999; Tryfinopoulou *et al.* 2002).

It needs to be noted that the identification and characterization of these SSOs in Mediterranean fish under different storage conditions has not been studied in depth. The use of the conventional phenotypic methods is not always effective to identify strains at species level (Stenstrom and Molin 1990), whereas molecular methods have been proved to be a powerful tool not only for identification at species level, but also for characterization at strain level (Ziemke *et al.* 1998; Reid and Gordon 1999; Venkateswaran *et al.* 1999; Fonnesbech-Vogel *et al.* 2000, 2005). However, so far, molecular fingerprinting analysis has been successfully applied to clinical isolates of *S. alga* (Fonnesbech-Vogel *et al.* 2000), whereas molecular phylogenetic analysis is not always efficient in tracking strains of *S. putrefaciens* in the food environment (Venkateswaran *et al.* 1999) in contrast to marine isolates of *Shewanella* species (Satomi *et al.* 2003, 2006). On the contrary, the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which is an advanced phenotypic tool standing between conventional and molecular methods, was great help for identification at species level (Vauterin *et al.* 1993; Vancanneyt *et al.* 1996). Indeed, the electrophoretic separation of cellular proteins is a sensitive technique that provides information on the similarity of strains at species level and it has successfully been used in *Shewanella* spp. isolates (Vauterin *et al.* 1993; Fonnesbech-Vogel *et al.* 1997).

The aim of the present study was to study the diversity of *Shewanella* population in *Sparus aurata* fish harvested in the Aegean Sea, as well as to elucidate the influence of fish storage conditions on the selection in *Shewanella* strains.

Material and methods

Fish preparation

Fresh, gilt head sea bream (*Sparus aurata*) was stored in ice immediately after capture in the Aegean Sea, Greece. The fish were kept in ice in a local fishery shop until they were bought within 6–8 h after capture and were transported in ice within 30–45 min from their purchase to the laboratory. They were then divided into six portions of 11 whole fish and were kept at 0, 10 and 20°C. The fish were stored in individual plastic bags, both aerobically and under modified atmosphere packaging (MAP; 40% CO_2 : 30% N_2 : 30% O_2) in a polyamide laminate packaging membrane (thickness 90 μm; gas permeability at 20°C and 50% RH, approx. 25, 90 and 6 cm^3 m^{-2} per day per bar for CO_2, O_2 and N_2, respectively), using a Henkovac 1900 packaging machine (Hectongen Bosch, The Netherlands). Samples were taken at appropriate time intervals to monitor microbial growth.

Microbiological sampling and analysis

A 25 g portion from the dorsal half of the fish was transferred in a stomacher bag (Seward Medical, London, UK); 225 ml of 0·1% (w/v) peptone water with salt (NaCl, 0·85%, w/v) was added and the suspension was homogenized for 60 s with a stomacher (Lab Blender 400, Seward Medical, London, UK). From the serial dilutions [in 0·1% (w/v) peptone water with NaCl, 0·85%, w/v] of fish homogenates, samples (1 ml) were inoculated into 10 ml of molten (45°C) Iron agar (IA; Oxoid code CM 867). After setting, a 10-ml overlay of the molten medium was added and the dishes incubated at 25°C for 3–5 days. Black colonies formed because of the production of H_2S were selected and enumerated. Three replicates of at least three dilutions were enumerated. All plates were examined visually for typical colony types and morphological characteristics associated with the growth medium.

Curve fitting

The growth data from plate counts were transformed to $\log_{10}$ values. The Baranyi model (Baranyi *et al.* 1993) was fitted to the logarithm of the viable cell concentration. For curve fitting, the in-house program DMFit, kindly provided by Dr J. Baranyi (Baranyi *et al.* 1993) (Institute of Food Research, Reading, UK) was used.

Purification and biochemical characterization of strains

From Iron agar Petri dishes, with an average of 30–50 colonies, colonies were selected according to their morphological characteristics (circular colonies, 1–4 mm in diameter, smooth, convex, with regular edges and black colour on Iron agar) and were purified by streaking into nutrient agar plates incubated at 25°C for 3–days. A total of 130 colonies were isolated, namely, 10 colonies from the initial microflora, 10 from the middle and 10 from the final day of each storage condition. Middle and final days were considered those where the population increased by 2·0–2·5 log CFU g^{-1} and by 3·5–4·0 log CFU g^{-1}. This corresponded to 5d, 2d and 1d (middle

day) and 10d, 3d and 2d (final day) at 0, 10 and 20°C in air, respectively, whereas under MAP 12, 5 and 2 days (middle day) and 16, 11 and 3 days (final days) at 0, 10 and 20°C, respectively.

All new isolates along with the type strains of *S. oneidensis* LMG19005^{TM}, *S. putrefaciens* ATCC8071^{TM} and *S. baltica* LMG2250^{TM} were then examined for physiological characteristics such as: Gram staining (Gregersen 1978), cell morphology on Tryptone Soya Yeast Agar (TSYA), flagellar arrangement (Mayfield and Einnis 1977), oxidase reaction (Kovacs 1956), catalase formation, aerobic and anaerobic breakdown of glucose for oxidative, fermentative or alkaline reaction (Hugh and Leifson 1953), reduction of trimethylamine oxide (TMAO) and production of hydrogen sulfide (Gram *et al.* 1987), ornithine decarboxylase (Falkow 1958), ammonia production from arginine (Thornley 1960), acid production from maltose (Cowan *et al.* 1993), production of lipase (Sierra 1957) and protease, egg yolk reaction, and growth at different temperatures with or without NaCl (Molin and Ternstrom 1986). Growth at 6% NaCl concentration was investigated in Trypticase Soy Broth (TSB; Difco), and in the absence of NaCl in TSB (Yoon *et al.* 2004).

The utilization of carbon sources was studied based on the method, which is described by Molin and Ternstrom (1982, 1986). The basal medium containing 0·1% (w/v) filter-sterilized single carbon sources was used. The following carbon sources were tested: D-arabinose, DL-carnitine, D-glucose, hydroxy-L-proline and D-mannitol (Serva, Heidelberg, Germany), arabitole, creatine, deoxycholate, D-galactonate, D-glucuronate, 4-hydroxy-benzoate, inosine, D-quinate and D-saccharate (Sigma, St Louis, MO, USA), meso-inositol and D-xylose (Merck, Darmstadt, Germany), malonate (Ferak Berlin, Berlin, Germany) and mucate (Fluka, Steinheim, Switzerland). Strains were grown overnight on nutrient agar at 25°C. Then, a colony was transferred to 10 ml of nutrient broth and incubated for 18 h at 25°C. Cells were collected aseptically by centrifugation at 4°C for 15 min at 13 518 ***g***, washed twice with physiological saline solution (NaCl, 0·85%, w/v), and finally resuspended on 1·0 ml of the same solution. Washed cells [$>10^6$ cells per millilitre – the estimation performed using MacFarland Standard (Ref. 70900 bioMerieux, St Louis, MO, USA)] were plated on microtitre plates containing basal medium with single carbon sources and incubated at 25°C. Growth was assessed on days 1, 4, 7, 12 and 14 by using a microtitre plate reader. Tests were performed in triplicate. A blank without an added carbon source and a standard with added glucose were also used as described by Drosinos (1994). Moreover, API 20E, 20NE and 50CH (bioMerieux) were used to test the utilization and fermentation of further carbon sources, according to the instructions of the manufactures.

Three representative isolates, namely LMBF-L1816 (=LMG 23023), LMBF-L2013 (=LMG 23024) and LMBF-L1716 (=LMG 23025), were deposited in the public BCCM/LMG Bacteria Collection (URL: http://www.belspo.be/bccm/lmg.htm).

SDS-PAGE analysis of whole cell proteins

Strains were subcultured in 9 ml of nutrient broth for 24 h at 25°C. They were then grown on nutrient agar for 48 h at 25°C. Cells were collected and washed with phosphate buffer (pH: 7·3; 10 mmol l^{-1}), containing NaCl, 0·8% (w/v). Cell extracts were prepared from approxi. 100 mg of bacterial cells (wet weight) and suspended in 1 ml of Tris-HCl buffer (pH 6·8; 62 mmol l^{-1}), containing 2% (w/v) sodium dodecyl sulfate, 5% (v/v), mercaptoethanol and 10% (v/v) glycerol. The cell suspension was heated for 10 min at 100°C, cooled on ice, and centrifuged at 4°C for 15 min at 13 518 ***g***. The supernatant (protein extract) was then stored at −20°C and used for the SDS-PAGE analysis (Toffin *et al.* 2004). Whole-cell protein extracts were prepared as described above. Registration of the protein electrophoretic patterns, normalization of the densitometric traces, clustering of strains by the Pearson product-moment correlation coefficient, and by the Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) analysis were performed as described by Pot *et al.* (1994) using the software package GelCompar (version 4·0; Applied Maths, Kortrijk, Belgium).

16S rRNA gene sequence analysis

Genomic DNA was extracted using the DNA isolation protocol of Niemann *et al.* (1997). A fragment of the 16S rDNA gene (corresponding with the positions 8-1541 in the *Escherichia coli* numbering system) was amplified by PCR using the conserved primers pA (5′ AGA GTT TGA TCC TGG CTC AG 3′) and pH (5′ AAG GAG GTG ATC CAG CCG CA 3′). PCR-amplified 16S rDNAs were purified using the NucleoFast® 96 PCR Cleanup Kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and purified using the Montage^{TM} SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA, USA). Sequencing was performed using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems). Nearly complete sequences were determined of strains L1816 (R-26018; 1497 bp), L2013 (R-26019; 1497 bp) and L1716 (R-26020; 1497 bp), using the eight sequencing primers listed in Coenye *et al.* (1999). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). Sequence similarities between the consensus sequences and small

Table 1 Data of H_2S-producing bacteria (enumerated on Iron agar), grown on *Sparus aurata* at different storage conditions [air and MAP (40% CO_2, 30% O_2, 30% N_2) at 0, 10 and 20°C]

	AIR					MAP (40%CO_2, 30% O_2, 30% N_2)				
Storage conditions	$Days^{-1}$ rate ± SE (rate)	Days lag ± SE (lag)	yEnd ± SE (yEnd)	SE (fit)	R^2	$Days^{-1}$ rate ± SE (rate)	Days lag ± SE (lag)	yEnd ± SE (yEnd)	SE (fit)	R^2
0°C	0·692 ± 0·074	1·878 ± 0·430	7·859 ± 0·163	0·235	0·98	0·237 ± 0·019	3·745 ± 0·618	6·882 ± 0·088	0·132	0·99
10°C	1·507 ± 0·081	–	8·909 ± 0·065	0·156	0·99	0·383 ± 0·047	–	7·544 ± 0·105	0·247	0·96
20°C	3·079 ± 0·360	–	8·707 ± 0·103	0·270	0·98	1·968 ± 0·114	–	8·685 ± 0·049	0·099	0·99

rate, growth rate; lag, lag time; yEnd, maximum final population; SE, standard error; fit, fitting curve and R^2, squared value.

ribosomal subunit sequences collected from the international nucleotide sequence library EMBL were calculated pairwise using an open gap penalty of 100% and a unit gap penalty of 0% with the software package BioNumerics version 3·5 (Applied Maths).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of strains LMBF-L1816 (=LMG 23023), LMBF-L2013 (=LMG 23024) and LMBF-L1716 (=LMG 23025) were deposited in the EMBL database (EMBL-EBI, Hinxton, Cambridge, UK) under the accession numbers AJ967026, AJ967027 and AJ967028, respectively.

Results

Changes in the population of H_2S-producing bacteria

The growth of H_2S-producing bacteria in fish stored under different packaging conditions is depicted in Table 1. The growth rate of H_2S-producing bacteria under both packaging conditions (Table 1) was higher at 20°C than the other two storage temperatures (0 and 10°C).

Characterization of *Shewanella* strains

According to the oxidase reaction and the oxidation/fermentation of glucose on Hugh and Leifson medium, 22 out of the 130 isolates were assigned to *Enterobacteriaceae*, *Aeromonas* and/or *Vibrio* and they were thus excluded from further study. From these 22 strains, 10 isolates originated from air and 12 from the modified atmosphere packaging conditions at 20°C.

The remaining 108 isolates were further analysed by the SDS-PAGE of whole cell protein (Fig. 1). Numerical analysis of their protein profiles showed that the isolates were separated into two distinct clusters A and B with 47% similarity. Cluster B was further subdivided into two subclusters B1 and B2, with 70% similarity. One strain could not be assigned to any of these groups. As discussed below, the clustering obtained by this method was in accordance to the grouping of the strains through the conventional phenotypic approach.

For this, the 108 new isolates, along with the type strains of *S. putrefaciens*, *S. oneidensis* and *S. baltica*, were characterized as follows: all isolates were Gram-negative, rod-shaped, nonspore-forming and motile. Cells grown on TSYA medium produced circular, smooth and convex colonies. On nutrient agar, the isolates showed a pinkish or brownish colouration that became darkest as time passed and colonies grew. All strains were oxidase and catalase positive and were unable to ferment glucose on Hugh and Leifson medium. All strains, grown at 4°C, without (0% NaCl) or with 6% NaCl, produced H_2S, ornithine decarboxylase, β-galactosidase, and reduced nitrate to nitrite, TMAO to trimethylamine and hydrolysed esculin and gelatin. None of them produced acid from maltose, luminescence and indole or displayed growth at 42°C. In addition, all strains assimilated D-quinate, arabitole and hydroxy-L-proline, whereas they were unable to utilize D-mannitol, creatine, inosine, meso-inositol, 4-hydroxy-benzoate, malonate, caprate and adipate. Moreover, they fermented ribose, esculine, 2-keto-gluconate and 5-keto-gluconate.

Differences among the 108 new isolates were observed concerning the utilization of other carbon sources as well as some biochemical properties (Table 2). It needs to be noted that some of the phenotypic characteristics found in this work are different, e.g. urease activity of *S. baltica*, gelatinase production of *S. putrefaciens* and *S. baltica*, ammonia production from arginine of *S. baltica*, from other similar studies, despite the fact that these data were derived from the same type strain. This observation is of great importance as it demonstrates the variability of possible outcomes derived by using different techniques. Similar variable results have been reported for

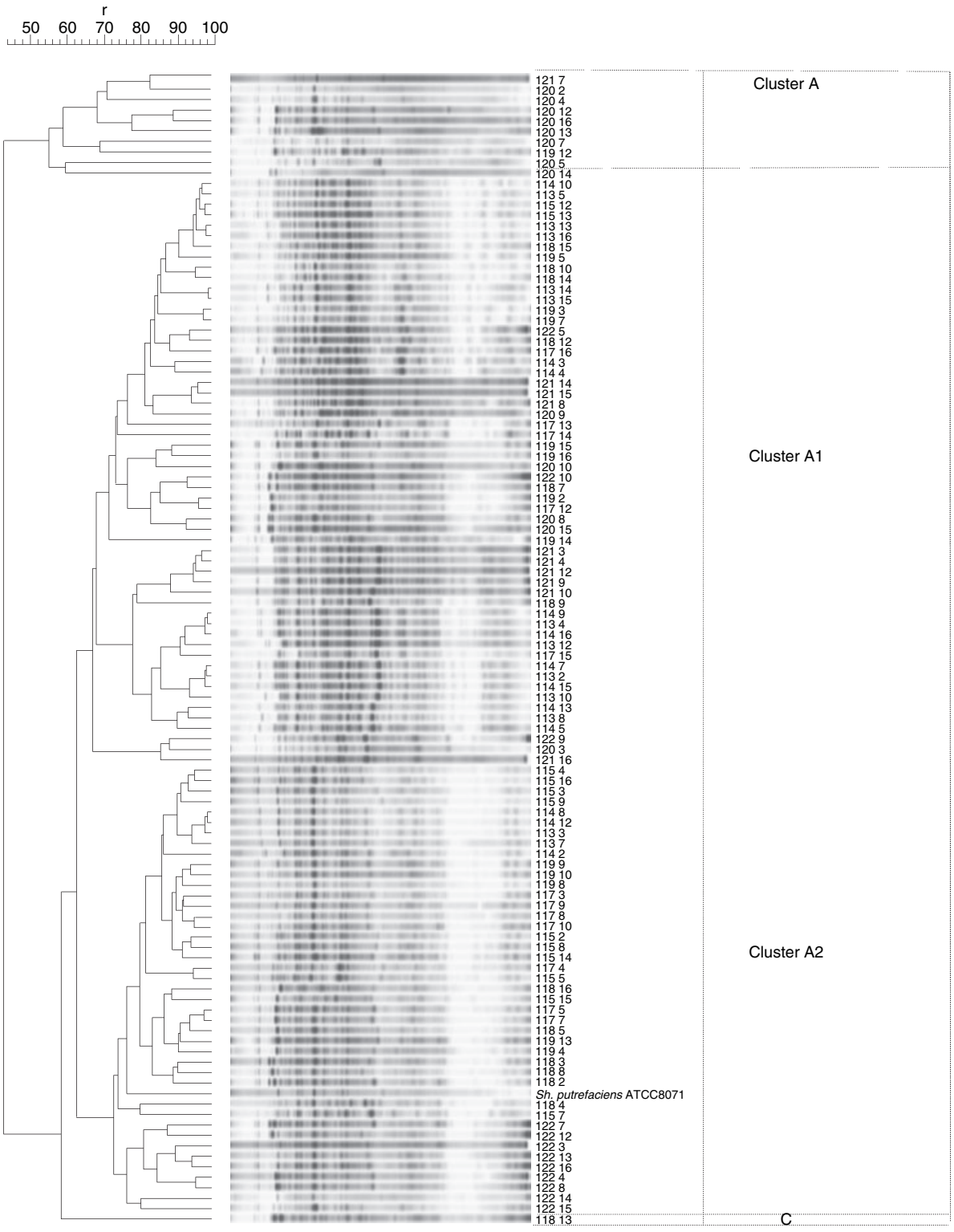


Figure 1 Clustering of SDS-protein electrophoregrams of 108 isolated strains of Shewanella spp.. The Pearson product-moment correlation coefficient and the UPGMA method were used. The horizontal scale represents percent similarities. The vertical scale recognizes the strains and clusters of Shewanella strains. The reproducibility of the SDS-PAGE technique was estimated by including duplicate runs of a single protein extract on separate gels ($r \geq 0{\cdot}94$).

Table 2 Phenotypic characteristics differentiating groups of *Shewanella* strains

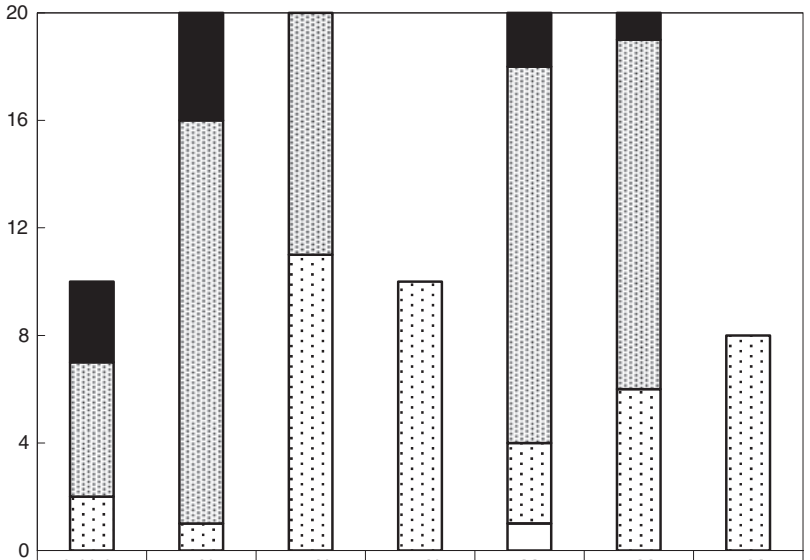
	Isolated strains				Type strains		
Characteristic/ Groups-Strains	A (*n* = 10, L2013)	B1 (*n* = 56, L1716)	B2 (*n* = 41, L1816)	C (*n* = 1)	*S. putrefaciens ATCC8071*	*S. oneidensis LMG19005*	*S. baltica LMG2250*
Reduction of							
Nitrite to nitrogen	0	0	0	–	–	–	+
Growth at							
37°C	0	0	0	+	+	+	–
Production of							
Ammonia from arginine	0	0	0	–	–	–	+
Protease	100	100	100	+	+	+	–
Lipase	100	92	100	+	–	–	+
Hydrolysis of							
Urea	100	100	95	+	–	–	+
Gelatin	100	90	100	+	+	+	–
On Hugh and Leifson medium							
Oxidative metabolism	0	0	100	+	–	–	+
Alkaline metabolism	100	0	0	–	+	–	–
Utilization of carbon sources							
D-Glucose	100	100	100	+	–	–	+
L-arabinose	100	100	100	+	–	–	–
Malate	100	100	100	+	–	–	+
D-Xylose	98	95	95	+	–	+	–
Sucrose	15	21	10	–	–	–	+
Gentiobiose	0	0	0	–	–	–	+
Cellobiose	0	0	0	–	–	–	+
Glycogen	0	0	0	–	–	–	+
D-mannose	0	0	0	–	–	–	+
Amygdaline	0	0	0	–	–	–	+
Deoxycholate	10	15	100	+	–	–	–
D-Saccharate	0	0	2	+	–	–	+
D-Glucuronate	0	100	0	–	–	–	–
N-acetyl-glucosamine	0	100	100	+	+	–	+
D-maltose	100	0	100	+	–	–	+
Gluconate	100	0	0	–	–	–	+
Citrate	100	0	0	–	–	–	+
Fermentation of			0				
Glucose	0	0	0	–	–	–	+
L-arabinose	0	0	0	–	–	–	+
D-lyxose	0	100	0	–	–	+	–
D-arabinose	0	100	0	–	–	–	–
D-xylose	0	100	0	–	–	+	–
L-xylose	0	100	0	–	–	+	–
N-acetyl-glucosamine	0	100	0	–	–	–	–

–, Negative reaction; +, Positive reaction.

S. putrefaciensT ATCC 8071. Indeed the hydrolysis of gelatin has been reported as positive (Fonnesbech-Vogel *et al.* 2005) as well as negative (Yoon *et al.* 2004). In the present study, this specific test has been carried out using API 20NE.

As mentioned before, these differences grouped the strains in a way highly correlated with the clustering obtained by the SDS-PAGE analysis. In fact, grouping based on the utilization of deoxycholate, D-saccharate and D-glucuronate, N-acetyl-glucosamine, D-maltose, gluconate and citrate, as well as the type of metabolism on the Hugh and Leifson medium (Table 2), was compatible to the clustering derived from the computerized numerical analysis of the protein electrophoregrams (Fig. 1).

Cluster A (Table 2) comprised 10 strains exhibiting alkaline metabolism on the Hugh and Leifson medium. In addition, these strains utilized D-maltose, gluconate and citrate. Cluster A contained nine strains isolated from



	Initial	0 Air	10 Air	20 Air	0 Map	10 Map	20 Map
A	3	$4^{*3m/1f}$	0	0	2^{2f}	1^{1m}	0
B1	5	$15^{7m/8f}$	$9^{4m/5f}$	0	$14^{8m/6f}$	$13^{7m/6f}$	0
B2	2	1^{1f}	$11^{6m/5f}$	$10^{5m/5f}$	$3^{2m/1f}$	$6^{2m/4f}$	8^{8f}
C	0	0	0	0	1^{1f}	0	0

Figure 2 Contribution, to each cluster, of Shewanella strains isolated from gilt head-seabream fish (Sparus aurata) stored in air and under MAP conditions at 0, 10 and 20°C. *, m/f are corresponding to the number of the strains, which are isolated from the middle (m) and final (F) day, respectively.

the initial microflora of fish maintained at 0°C under both storage conditions, and one originated from fish stored at 10°C under MAP (Fig. 2). Cluster B1 (Table 2) included 56 strains exhibiting neither oxidative nor alkaline metabolism on the Hugh and Leifson medium, and utilizing D-glucuronate and N-acetyl-glucosamine. Strains belonging to cluster B1 fermented D-lyxose, D-arabinose, D-xylose and N-acetyl-glucosamine. They were isolated from all storage conditions except those at 20°C (Fig. 2). Another 41 strains were grouped in cluster B2 (Table 2). They exhibited oxidative metabolism on the Hugh and Leifson medium and utilized deoxycholate as well as N-acetyl-glucosamine and D-maltose. Strains of cluster B2 were isolated from all storage conditions (Fig. 2). Finally, one strain isolated from fish stored at 0°C under MAP, could not be assigned to any of the clusters described above. It exhibited oxidative metabolism on the Hugh and Leifson medium and utilized deoxycholate, D-saccharate, N-acetyl-glucosamine and D-maltose.

On the contrary, strains of all three clusters shared common traits as far as reduction of nitrite to nitrogen, growth at 37°C, production of ammonia from arginine, protease and lipase activities, and hydrolysis of urea and gelatine are concerned (Table 2). However, these common traits, as well as the differentiating features highlighted above, did not allow the assignment of any of the three clusters to the *S. putrefaciens*, *S. oneidensis* and *S. baltica* species (Table 2). Thus, three representative strains of the major clusters A (LMBF-L2013), B1 (LMBF-L1716) and B2 (LMBF-L1816) were selected for determination of the nearly complete 16S rRNA gene sequence. Interestingly, the three strains showed over 99·4% sequence similarity among each other. The phylogenetic tree constructed by the neighbour-joining method is shown in Fig.3. These results suggest that the isolated strains belong to the genus *Shewanella*, whereas high similarities (>97%) were found with *S. baltica* LMG2250^{TM}, *S. putrefaciens* ATCC8071^{TM} and *S. oneidensis* LMG19005^{TM}.

Discussion

It is well established that H_2S-producing bacteria play an important role in fish spoilage and has already been characterized as the main spoilage organism in cod from North Sea (Europe) after storage in air (Gram and Dalgaard 2002). Fonnesbech-Vogel *et al.* (2005) characterized *S. baltica* as the main H_2S-producing species of Baltic fish microflora. Later on, more *Shewanella* species (*S. hafniensis* and *S. morhuae*) have been isolated from fish originated from the same area (Satomi *et al.* 2006). In the microflora of fish (boque) originated from the Mediterranean Sea, *Shewanella* species was among the main spoilage organisms (Koutsoumanis and Nychas 1999), characterized as H_2S-producing bacteria. In the packed fish from the aforementioned geographical areas, cod and seabream, respectively, strains, which are characterized as

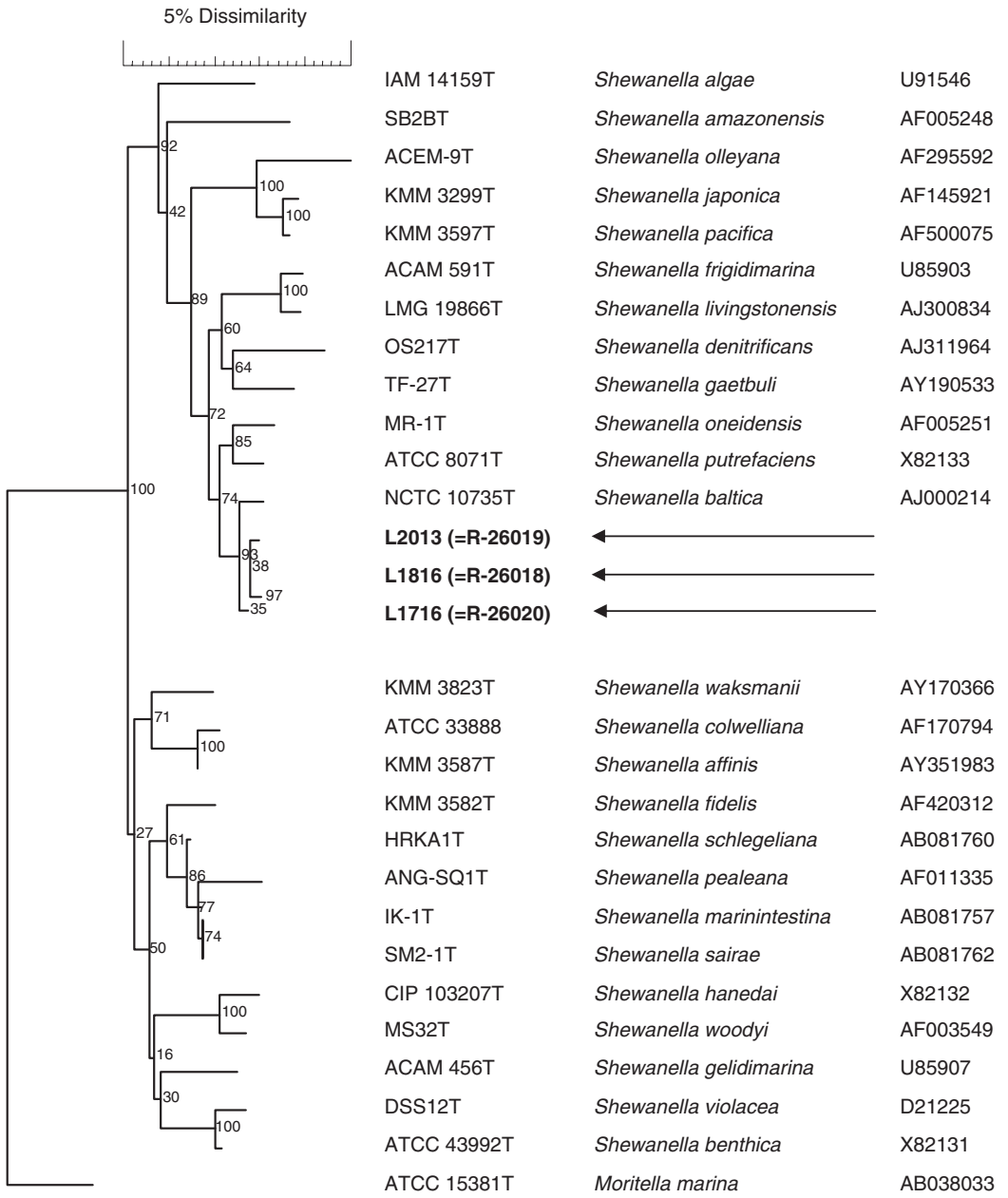


Figure 3 Phylogenetic tree obtained by neighbour-joining analysis of 16S rRNA sequences showing the positions of the isolates L2013, L1816, L1716 and related organisms. Strains and accession numbers are indicated.

S. putrefaciens, have also been found to be members of the spoilage microflora (Drosinos and Nychas 1997; Gram and Dalgaard 2002).

Although the role of *Shewanella* spp. in fish spoilage is well documented, the phylogenetic relationships among known strains are still an open issue for the scientific scrutiny. For over two decades, it has been known that *S. putrefaciens* is a genetically heterogeneous species. These conclusions were based upon the wide variation in G + C content (44–54%), the results of DNA–DNA re-association studies and numerical taxonomic investigations (Levin 1972; Owen *et al.* 1978; Semple *et al.* 1989; Stenstrom and Molin 1990; Fonnesbech-Vogel *et al.* 2000). In the late 1970s, Owen *et al.* (1978) showed that the species comprised at least four clearly separated genomic groups, I to IV. Later on, the study of Semple *et al.* (1989), using DNA–DNA similarity and 5S rRNA hybridization analysis, confirmed these

results. One of the *S. putrefaciens* genomic groups (IV) has been meanwhile reclassified as a new species; i.e. *S. algae* (Simidu *et al.* 1990), which included the most halotolerant members of the genus, the Fe^{3+} reducing organisms, as well as most of the clinical isolates (Nozue *et al.* 1992; Fonnesbech-Vogel *et al.* 1997; Khashe and Janda 1998). In spite of the intraspecific diversity of *S. putrefaciens*, the three other genomic groups were classified into the same species until the late 1990s. In 1998, Ziemke *et al.* (1998) showed that members of Owen's genomic group II were phenotypically, genotypically and phylogenetically distinct enough to be classified as a new species, *S. baltica.* Venkateswaran *et al.* (1999) based on a polyphasic approach defined Owen's group III strains as a new species, *S. oneidensis*. According to the authors, Owen's group I strains that are highly related to the type strain ATCC 8071 should be considered as the true *S. putrefaciens*.

It is well known that the clustering of *Shewanella* isolates may depend not only on the method of classification but also on the origin of the strains (Stenstrom and Molin 1990; Fonnesbech-Vogel *et al.* 2000). It should be noted that in the present study no mesophilic strains were found among the strains recovered from the Mediterranean *Sparus aurata* fish (Table 2), and that the selection of *Shewanella* strains was clearly influenced by the storage temperature (Fig. 2). Indeed, strains of subclusters A and B1 originated from fish maintained at lower storage temperatures (0 and 10°C), whereas strains of subcluster B2 were isolated from all tested storage temperatures, independently the packaging conditions, aerobic or MAP.

Data generated in the present study, and in particular the phenotype, which is based on key characteristics of the family (Stenstrom and Molin 1990; Ziemke *et al.* 1998; Ivanova *et al.* 2004), infer that the new isolates belong to *Shewanellaceae*. The results of the conventional phenotypic characterization (Table 2) were in full accordance to those reached by whole-cell protein profiles (Fig. 1), as both analyses separated the *Shewanella* isolates into three distinct subgroups (A, B1 and B2). Their phenotypic characteristics discriminate these subgroups from the already defined species of *Shewanellaceae* and more specifically to the closest phenotypically related *S. baltica* (Ziemke *et al.* 1998), *S. oneidensis* (Venkateswaran *et al.* 1999) and *S. putrefaciens* (Lee *et al.* 1977**).** On the contrary, the high 16S rRNA gene sequence similarity of >99·4% among representative isolates of the major clusters A, B1 and B2 indicates that all isolates are genomically highly related. However, significant 16S rRNA gene sequence similarities (>97%) with *S. baltica* LMG2250^{TM}, *S. putrefaciens* ATCC8071^{TM} and *S. oneidensis* LMG19005^{TM} do not provide a clear genomic evidence to which of these species the strains belong (Stackebrandt and Goebel 1994). Thus, whether these new isolates represent a new species or they just confirm the high heterogeneity of *Shewanellaceae* remains to be revealed. For this, labour-intensive DNA–DNA hybridization experiments or *gyr*B gene sequence analysis (Venkateswaran *et al.* 1999) are required to prove unequivocally the species identity, or indicate possible genomic heterogeneity among the clusters.

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