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A survey on bacteria isolated as hydrogen sulfide-producers from marine fish

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ABSTRACT

Aim of the work was to identify sulfide-producers isolated from tuna and swordfish and to evaluate some physiological characteristics, particularly those that could be related to spoiling potential.

16S rDNA sequencing revealed *Shewanella baltica* as the main species, followed by *Serratia* spp. and other *Shewanella* species, while RAPD- and rep-PCR analyses indicated the presence of several biotypes. *Shewanella baltica* and *Shewanella putrefaciens* showed rapid growth at 4 and 8 °C, production of TMA and H_2S, amino acids decarboxylation and proteolytic activity also at refrigeration temperatures, therefore being potentially able to modify texture and sensory characteristics of finfish. Extracellular DNAse activity and growth in presence of high salt concentrations can provide a competitive advantage in unfavourable environments.

Our data provide new insights into specific metabolic features of *Shewanella* spp., rarely studied before, such as extracellular DNAse activity and amino acid decarboxylating activity. Moreover, our results highlight the clear necessity of more specific media and research methods to count H_2S-producing bacteria.

1. Introduction

Fresh fish is highly susceptible to spoilage, which can be caused both by chemical reactions and microbial growth. Not all the microorganisms growing in raw fish participate in the spoilage process, whereas, the Specific Spoilage Organisms (SSOs) are generally present in low numbers and differ depending on seafoods (Gram & Dalgaard, 2002). To lengthen finfish shelf-life, it is necessary to identify and characterize the bacteria able to spoil fish products, considering their metabolic traits and their physiological features in terms of adaptability to different environmental conditions.

The most common spoilage bacteria reported in fish and fish products are *Shewanella putrefaciens*, *Photobacterium phosphoreum*, lactic acid bacteria (Rudi, Maugesten, Hannevik, & Nissen, 2004) and *Pseudomonas* spp. (Gennari, Tomaselli, & Cotrona, 1999). In particular, microorganisms of the genus *Shewanella*, and namely the species *S. putrefaciens* and *S. baltica*, are widespread in marine environment and therefore are commonly isolated from fish. Both the species are psychrotrophic (Vogel, Venkateswaran, Satomi, & Gram, 2005) and able to grow on ice-stored fish. *Shewanella putrefaciens* can cause spoilage in both fresh and packed fish, producing off-odours even at low cells numbers (Dalgaard, 1995). The even greater diffusion of fish products packed in MAP requires new efforts in controlling *Shewanella* growth and spoilage.

Until recent years, Gram negative, catalase and oxidase-positive bacteria, able to produce hydrogen sulfide and TMA, were commonly identified as *S. putrefaciens*. In 2005, Vogel and collegues demonstrated that these characteristics, associated with other metabolic traits, only allowed to define the affiliation to the genus *Shewanella*, but were not sufficient to define the species. On the basis of further phenotypic tests, they concluded that *S. baltica* and not *S. putrefaciens* was the most represented species among H_2S producers in Danish ice-stored fish, constituting only a minor part of the initial microbial population of finfish and becoming dominant during refrigerated storage, up to 10^7–10^9 cfu g^{-1} (Vogel et al., 2005). The species *S. putrefaciens* has been then split into two species, *S. putrefaciens* and *S. baltica*, in 1998 (Ziemke, Höfle, Lalucat, & Rossello-Mora, 1998), and in many papers, bacteria with the characteristics listed above are commonly described as "*S. putrefaciens*-like" (Gram & Dalgaard, 2002).

Very few papers have been published on the metabolic and spoiling characteristics of *Shewanella* and other H_2S-producing bacteria. For this reason, the aim of this study was to identify and characterize sulfide-producing bacteria, isolated from raw tuna fish

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and swordfish samples. In particular, characterization was aimed to study some physiological aspects, such as salt resistance, growth at different temperatures and specific metabolic features, potentially involved in spoiling, i.e. production of spoilage markers such as trimetylamine (TMA) and hydrogen sulfide, DNAse, proteolytic and amino acids decarboxylating activity.

2. Materials and methods

2.1. Bacterial strains, culture media, growth conditions and preliminary tests

Fifty strains were isolated as H_2S-producers from retail samples of red tuna and swordfish steaks.

Three samples of red tuna and four of swordfish were weekly collected in November, as fresh fish (T0), refrigerated in aerobiosis. Only one swordfish sample was stored for three days in the same conditions, before being analysed. Samples were previously analysed and sulfide-producers load, determined on Kligler Iron Agar (Oxoid, Basingstoke, UK) incubated at 25 °C, was between 3.15 and 4.64 Log cfu g^{-1} in tuna, and between 1.0 and 5.11 Log cfu g^{-1} in swordfish.

Shewanella putrefaciens ATCC 49138 was used as reference strain.

Strains were maintained as frozen stocks at −80 °C in presence of glycerol 20% (v/v) as cryoprotective agent. Tryptone Soy Broth (TSB) and Tryptone Soy Agar (TSA, Oxoid), both added of 0.5% NaCl (v/v), were used to reactivate and cultivate the strains at 25 °C for 24 h.

All strains were tested for the following characteristics: KOH 3% (w/v) test (Gregersen, 1978), catalase test (3% H_2O_2), motility and cell morphology determination (optical microscopy).

All the chemicals used in this study were from Sigma–Aldrich, Milan, Italy.

2.2. Molecular identification and characterization

2.2.1. DNA extraction

Overnight cultures, grown in TSB modified with 0.5% NaCl (TSBm) at 25 °C, were washed twice in Phosphate Buffer Saline (PBS, 8.5 g l^{-1} NaCl, 0.93 g l^{-1} KH_2PO_4, 0.15 g l^{-1} NaOH) and centrifuged at 1.2 g for 3 min. Cells were lysed with 200 μl of lysis buffer (0.05 mol l^{-1} NaOH, 0.25% w/v SDS) at room temperature for 15 min. The samples were then centrifuged at 13,000 rpm for 10 min, and the supernatants were purified with immunoaffinity column GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA), according to manufacturer's instructions.

2.2.2. Molecular fingerprinting

For RAPD fingerprinting, two different amplifications for each strain were performed using the primers M13 (5′- GAG GGT GGC GGT TCT -3′) (Stenlid, Karlsson, & Hogberg, 1994) and OPA18 (5′- GAC GCT AGT G-3′) (Vogel et al., 2000). DNA was amplified as previously described for M13 (Serio, Paparella, Chaves López, Corsetti, & Suzzi, 2007) and OPA18 (Vogel et al., 2000).

Genomic DNA was also amplified using the primer $(GTG)_5$ (5′- GTG GTG GTG GTG GTG -3′) (Scheirlinck et al., 2007), to obtain rep-PCR profiles. The amplification was performed in the following mix reaction: Buffer 1X, DNTPs 0.2 mmol l^{-1}, primer $(GTG)_5$ 1.0 μmol l^{-1}, Taq polymerase 5U/100 μl, $MgCl_2$ 3.4 mmol l^{-1}, and 2.5 μl of template were added. Thermal cycling conditions were as follows: 95 °C for 7 min, 30 cycles of 90° for 30 s, 40 °C for 1 min, 65 °C for 8 min, then 90 °C for 30 s, 40 °C for 1 min, and 65 °C for 16 min.

PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel in 1X TAE (Tris-Acetate-EDTA) buffer. After staining in ethidium bromide, the images were analysed using Fingerprinting II, Informatix Software (Bio-Rad). Grouping of RAPD- and rep-PCR profiles was performed by the Pearson product moment correlation coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) cluster algorithm. All profiles obtained with both methods were analysed together as a single data set, by calculating the average matrix from the separate similarity matrices of the different primers, to obtain a single dendrogram.

2.2.3. 16S rDNA amplification, sequencing and species attribution

The isolates were identified by means of 16S rDNA gene sequencing. The primers 16S for (5′- CAG GCC TAA CAC ATG CAA GTC -3′) and 16S rev (5′- GGG CGG AGT GTA CAA GGC -3′) were used for 16S rDNA amplification (Mahmoud et al., 2004). The amplification products were purified by means of GFX™ PCR DNA and Gel Band Purification Kit, following manufacturer's recommendations.

Gene sequencing was performed by BMR-Genomics (Padua, Italy), and the resulting sequences were compared with those in the GenBank database (http://www.ncbi.nlm.nih.gov), by means of Basic Local Alignment Search Tool (BLAST) software.

2.3. Physiological characterization

Growth at different temperatures has often been considered a discriminant test to distinguish different species within the genus *Shewanella*. Overnight cultures were inoculated (1% v/v) in TSBm and incubated up to 60 days at 4, 8, 11, 20, 25, 30, 37 and 42 °C, to evaluate growth. In the same way, cells were inoculated in TSBm, added with 4, 6, 8, 10 and 12% NaCl, and were incubated at 25 °C for 7 days, to determine their salt tolerance. Results were expressed as days necessary to reach an $O.D._{600}$ value of 0.420–0.470, approximately corresponding to 9.65–9.90 Log cfu ml^{-1}.

API 20NE galleries (BioMerieux®, Mercy-l'Etoile, France) were used to test biochemical characteristics. They were prepared according to manufacturer's instruction and incubated at 25 °C for 24 h.

Extracellular DNAse activity was assessed on DNAse Test Agar (Beckton Dickinson Italia, Milan, Italy). After 24 h of incubation at 25 °C, HCl 1N was poured onto plates, and colonies surrounded by a clarifying halo were considered positive.

TMAO reduction and H_2S production were estimated according to Gram, Trolle, and Huss (1987), substituting resazurin with 0.025% methylene blue. TMAO reduction was evidenced by colour modification from blue to pink, while H_2S production from sodium thiosulfate and/or L-cysteine produced black precipitates. Incubation was performed at 25 °C for a maximum of 72 h.

Iron Agar base (IA) was prepared according to Gram et al. (1987) to evaluate isolates capability to produce H_2S in three media differing in sulfur source: 0.03% sodium thiosulfate in medium IA1, 0.04% L-cysteine in IA2, 0.03% sodium thiosulfate and 0.04% L-cysteine in IA3. Inoculated media were incubated at 25 °C for five days.

Decarboxylating activity on amino acids tryptophan, phenylalanine, histidine, ornithine, lysine and tyrosine was assessed on Petri dishes containing the following basal medium: 0.5% peptone, 0.5% Lab Lemco, 0.05% dextrose, 0.001% bromocresol purple, 0.0005% pirydoxal-5-phospate, pH 6.8. Each amino acid (1%) was individually added to the basal medium. The medium without amino acids was considered as control. Plates were spotted with the isolates and then incubated at 25 °C for 5 days in anaerobic conditions. Positive colonies were surrounded by a dark violet halo.

Proteolytic activity was evaluated according to Cd-nynhydrin method proposed by Folkertsma and Fox (1992). To reproduce environmental conditions of isolates, Marine Broth supplemented with peptone 2.0% (w/v) was used. This medium (5 ml) was