Food Bioprocess Technol (2011) 4:907–918
DOI 10.1007/s11947-010-0441-2

REVIEW PAPER

# Safety Assessment of Fresh and Processed Seafood Products by MALDI-TOF Mass Fingerprinting

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Received: 5 August 2010 / Accepted: 19 September 2010 / Published online: 5 October 2010


**Abstract** Foodborne intoxications caused by the consumption of fish and other products of marine origin contaminated with bacterial pathogens are an ever-present threat, either due to bacteria and/or its metabolites. In addition, the rapid spoilage of seafood due to microbial activity, results in high economic losses. The development of the microbiota in seafood products depends on the microbiological ambience of capture, processing and storage, and the applied preservation method. Thus, pathogenic and spoilage bacterial species in seafood may come from the indigenous microbiota of the aquatic ambience or are introduced by contamination during processing. Rapid and accurate bacterial species identification is essential for an effective control program to ensure safety and quality of either processed or minimally processed seafood. In the present work, matrix-assisted laser desorption ionization-time of flight mass spectrometry was successfully applied to identify 26 bacterial strains isolated from fresh fish and processed seafood samples. The approach was based on the comparison of unknown spectra to a reference spectral library and demonstrated to be a fast and accurate technique for bacterial species differentiation, which can be used for the rapid identification of foodborne pathogens and spoilage bacteria potentially present in products of marine origin.

**Keywords** Seafoodborne pathogens · Seafood spoilage · Histamine fish poisoning · Seafood safety and quality assessment · Bacterial species identification · MALDI-TOF MS

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## Introduction

The consumption of fish and seafood is increasing, making the industry of fishing and aquaculture one of the largest international trading food commodities. Quality and safety of seafood is an important issue for the food industry, since seafood is the most frequent source of food poisoning, causing diseases with varying degrees of severity, ranging from mild indisposition to chronic or life-threatening illness (Iwamoto et al. 2010). In this sense, histamine poisoning is one of the most prevalent seafood intoxications, being induced by the consumption of fish with high levels of histamine, produced by mesophilic bacteria in inappropriately chilled seafood (Kim et al. 2003; Lehane and Olley 2000). Furthermore, the industry of fishing and aquaculture is confronted to high economic losses due to the rapid spoilage of seafood (Huss 1995; Rodriguez et al. 2003). The main cause of seafood spoilage is the growth and metabolism of bacteria, due to the ability to form volatile substances that cause off-odors (Fonnesbech Vogel et al. 2005; Gram 1992; Gram and Dalgaard 2002).

The microbial load in a product of marine origin is closely related to the conditions of the aquatic ambience and the initial level of bacterial contamination, as well as to

the applied preservation method, which influences the growth and survival of bacteria (Calo-Mata et al. 2008). In this sense, foodborne pathogenic, as well as spoilage bacteria can already be present in the indigenous microbiota, or are introduced to the seafood products by contamination during processing (Huss et al. 2000). The indigenous microbiota of fresh seafood contains mainly psychrotrophic, Gram-negative bacteria, such as *Aeromonas* spp., *Pseudomonas* spp., and *Shewanella* spp. (Gram and Dalgaard 2002). In general, pathogenic species are present in low concentrations in fresh seafood and spoilage bacteria overgrow the pathogenic ones, thus being more rapidly spoiled than becoming toxic (Feldhusen 2000). However, in seafood products coming from warm, coastal waters, the consumption of raw products and shellfish implies an elevated health risk due to the presence of toxin-producing *Vibrio* spp. (Eja et al. 2008; Lhafi and Kühne 2007). The growth of the normal psychrotrophic microbiota is inhibited by a light preservation and/or the storage under anaerobic conditions, favoring the growth of *P. phosphoreum* and Gram-positive bacteria (Gram and Huss 1996; Paludan-Müller et al. 1998). In addition, the proliferation of *Clostridium botulinum* and its very potent toxins are of concern in seafood that have been packaged under anaerobic conditions, since it is a naturally occurring marine organism that is able to grow at refrigeration temperatures (Feldhusen 2000). Furthermore, contamination of the aquatic environment by humans and/or animals can lead to the presence of *Enterobacteriaceae* (Ward 2001), including human pathogens, such as *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. that can cause serious foodborne intoxications. Nevertheless, most microbial contamination was found to occur during processing, such as filleting and subsequent handling prior to packaging. Some important food pathogens, such as *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*, are difficult to eliminate from the food-processing chain due to their ubiquitous character (Feldhusen 2000; Jay et al. 2005). To reduce the number of viable pathogens, seafood products are often subjected to a heat treatment after packaging or before consumption. Remarkably, such heat treatments do not destroy potential toxins and spores of spore-forming bacteria, such as *Bacillus* spp. and *Clostridium* spp. (Gram et al. 2002; Huss et al. 2000).

In order to control and minimize the microbiological hazard of foodborne pathogens and to predict and enhance shelf-life of seafood products, pathogenic and spoilage bacteria have to be identified in a rapid and unequivocal way. Traditionally, bacterial species have been identified by classic tools relying on culturing processes coupled to morphological, physiological, and biochemical characterization. In the last decades, the progress of microbiological identification turned to more rapid and sensitive methods, including miniaturized biochemical systems, antibody-based assays, and DNA-based methods, together with important advances in bioinformatic tools. Recently, the development of rapid and high sensitive techniques, such as real-time PCR, DNA microarrays, and biosensors, provoked the replacement of traditional culturing methods in the field of bacterial identification in clinical diagnostics, as well as in the food sector (Feng 2007; Mohania et al. 2008; Neethirajan and Jayas 2010). Furthermore, Fourier transform infrared spectroscopy (FT-IR) has been described as a new method for rapid and reliable bacterial identification (Al-Qadiri et al. 2008; Kuhm et al. 2009). At the same time, proteomic tools, such as mass spectrometry were introduced for the identification of microorganisms (Klaenhammer et al. 2007; Russell 2009). Several authors reported matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis to be a very fast and simple method for bacterial differentiation, due to the high specific spectral profiles (named fingerprints), obtained by this technique (Giebel et al. 2010; Lay 2001; van Baar 2000). Bacterial identification by MALDI-TOF MS fingerprinting is based on the comparison of the spectrum of an unknown strain to a reference spectral library. Many works have been done in constructing spectral databases of a huge number of bacterial strains (Dare 2006; Degand et al. 2008; Erhard et al. 2008; Keys et al. 2004). However, the critical challenge of these techniques is the limited availability of such reference databases. Furthermore, most studies in bacterial differentiation by MALDI-TOF MS fingerprinting are targeted at clinical diagnostics of bacterial strains associated with human infectious diseases, but few work has been done in the field of microbial food analysis (Mazzeo et al. 2006). In our laboratory, we constructed a library of mass spectral fingerprints of the main pathogenic and spoilage bacterial species, potentially present in seafood products (Böhme et al. 2010; Fernández-No et al. 2010). The aim of the present study was the application of the technique of MALDI-TOF MS fingerprinting for the identification of bacterial strains isolated from fresh and processed seafood products, on the base of the previously created reference spectral library.

## Materials and Methods

### Bacterial Strains and Culture Media

For this work, 26 bacterial strains were selected from the laboratory intern collection that included bacterial strains isolated from seafood (Table 1). In the case of fresh fish samples, stored on ice, only bacterial strains that showed positive results in proteolytic medium (Ben-Gigirey et al. 2000) and in the medium of Niven (Niven et al. 1981) were

**Table 1** Bacterial strains isolated from fresh and processed seafood samples

| Code | Bacterial species | Isolation source | Preservation method |
|---|---|---|---|
| 15MF | *Stenotrophomonas maltophilia* | Albacore | Iced |
| 25MC6 | *Stenotrophomonas maltophilia* | Albacore | Iced |
| Sard1 | *Proteus vulgaris* | Sardine | Iced |
| Sard2 | *Proteus vulgaris* | Sardine | Iced |
| Sard3 | *Proteus vulgaris* | Sardine | Iced |
| Sard4b | *Proteus vulgaris* | Sardine | Iced |
| Seab02 | *Pseudomonas syringae* | Seabream | Iced |
| Seab03 | *Pseudomonas fragi* | Seabream | Iced |
| Seab22 | *Pseudomonas fragi* | Seabream | Iced |
| Seab23 | *Pseudomonas fragi* | Seabream | Iced |
| Seab06 | *Stenotrophomonas maltophilia* | Seabream | Iced |
| Seab08 | *Stenotrophomonas maltophilia* | Seabream | Iced |
| Turb32 | *Pseudomonas fragi* | Turbot | Iced |
| Turb28 | *Pseudomonas fluorescens* | Turbot | Iced |
| Turb43 | *Pseudomonas fragi* | Turbot | Iced |
| Turb46 | *Pseudomonas fluorescens* | Turbot | Iced |
| Turb52 | *Pseudomonas fluorescens* | Turbot | Iced |
| Turb64 | *Pseudomonas fluorescens* | Turbot | Iced |
| Proc3T9 | *Bacillus cereus* | Processed seafood | Vacuum packed, mild heat treatment |
| ProcB2 | *Bacillus cereus* | Processed seafood | Vacuum packed, mild heat treatment |
| Proc721 | *Bacillus subtilis* | Processed seafood | Vacuum packed, mild heat treatment |
| ProcB6c | *Bacillus subtilis* | Processed seafood | Vacuum packed, mild heat treatment |
| Proc6a | *Bacillus subtilis* | Processed seafood | Vacuum packed, mild heat treatment |
| Proc6b | *Bacillus subtilis* | Processed seafood | Vacuum packed, mild heat treatment |
| Proc6c | *Bacillus subtilis* | Processed seafood | Vacuum packed, mild heat treatment |
| Proc7T6 | *Serratia marcescens* | Processed seafood | Vacuum packed, mild heat treatment |

isolated. In contrast, the strains isolated from processed seafood were part of a shelf-life study of the vacuum-packed seafood products that were subjected to a mild heat treatment (Table 1).

The frozen stored strains were reactivated in brain-heart-infusion (BHI; Becton, Dickinson and Company, Le Pont de Claix, France) and incubated for 24 h at 30 °C. Afterwards, bacterial cultures were grown on plate-count-agar (PCA; Oxoid, Hampshire, England) at 30 °C and single colonies were isolated.

The identification of the isolated strains by genetic tools was obtained by sequencing a fragment of the 16S rRNA and searching for sequence homologies among published reference sequences with the BLAST tool of the National Center for Biotechnology Information. Bacterial cells of pure overnight cultures in BHI were lysed and the total DNA was extracted and purified by means of the DNeasy Tissue Mini Kit (Qiagen, Valencia, CA, USA), as described elsewhere (Campos et al. 2006). A fragment of the 16S rRNA gene was amplified by PCR using the universal primer pair P8FPL/P806R (McCabe et al. 1999) and the products were sequenced using an BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) as described by Böhme et al. (2010).

## Proteomic Analysis by MALDI-TOF-MS

### *MALDI Sample Preparation*

For proteomic analysis, the bacterial strains were grown on PCA and incubated for 24 h at 30 °C. One loopful (1 μL loop) of each bacterial culture was harvested in 100 μL of a solution containing 50% acetonitrile (ACN; Merck, Darmstadt, Germany) and 1% aqueous trifluoroacetic acid (TFA; Acros Organics, NJ, USA) and mixed by vortexing. After centrifugation, the supernatant was transferred into a new tube and stored at −20 °C until analysis. Two extractions per sample were carried out. A 1-μL aliquot of the sample solution was mixed with 10 μL of the matrix solution: saturated α-cyano-4-hydroxycinnamic acid (α-CHCA; Sigma-Aldrich, Saint Louis, MO, USA) in 50% ACN and 2.5% aqueous TFA. A 1-μL aliquot from the sample/matrix solution was manually deposited onto a stainless steel plate and allowed to dry at room temperature.

*Spectra Acquisition*

Mass spectra were obtained using a Voyager DE STR MALDI-TOF Mass Spectrometer (Applied Biosystems, Foster City, CA, USA) operating in linear mode, extracting positive ions with an accelerating voltage of 25,000 V and a delay time of 350 ns. The grid voltage and guide wire were set to 95% and 0.05% of the extraction voltage, respectively. Spectra taken in the *m/z* range of 1,500–15,000 were obtained in ten different regions of the same sample spot and each was the result of the accumulation of at least 1,000 laser shots. Each of the two extracts was measured in duplicate, ultimately acquiring four spectra for each bacterial strain. The spectra were externally calibrated using a mixture of 2 pmol/μL oxidized B chain of insulin and 2 pmol/μL bovine insulin (Sigma-Aldrich).

*Data Analysis*

Mass spectra were analyzed with the DataExplorer® software (Version 4.0.0.0), baseline corrected, noise filtered and data lists containing *m/z* values were extracted from mass spectral data, including signals with relative intensities higher than 2%. For analysis, the *m/z* interval of 2,000–10,000 was considered due to the good reproducibility of the spectral profiles within that range. Peak mass lists were further processed with the free available web-based application SPECLUST (http://bioinfo.thep.lu.se/speclust.html) (Alm et al. 2006), extracting representative peaks present in all four spectra acquired for each sample. This web interface calculates the mass difference between two peaks taken from different peak lists and determines if the two peaks are identical after taking into account a certain measurement uncertainty ($\sigma$) and peak match score ($s$). Next, spectra of the strains isolated from commercial seafood samples were compared to a reference library that was created in our laboratory in previous studies (Böhme et al. 2010; Fernández-No et al. 2010). The library included mass spectral data of the main pathogenic and spoilage bacterial species potentially present in seafood. Reference strains used to create the library are indicated in Table 2. Final peak mass lists were compared with the web application SPECLUST, whereas a mass was considered shared between two spectra if the peak match score was larger than 0.7, corresponding to a width in the peak match score of 5 Da. Furthermore, the clustering option, also available on the web interface SPECLUST, was used to cluster the mass lists of the strains isolated from seafood

**Table 2** Reference bacterial strains used to establish the MALDI-TOF MS library of seafood pathogenic and spoilage bacteria

| Bacterial species | Strain ID | Bacterial species | Strain ID |
|---|---|---|---|
| *Acinetobacter baumanii* | ATCC 15308 | *Pantoea agglomerans* | ATCC 27155 |
| *Aeromonas hydrophila* | ATCC 7966 | *Photobacterium damselae* | ATCC 33539 |
| *Bacillus cereus* | ATCC 14893 | *Photobacterium phosphoreum* | CECT 4172 |
| *Bacillus licheniformis* | ATCC 14580 | *Proteus mirabilis* | ATCC 14153 |
| *Bacillus megaterium* | ATCC 25848 | *Proteus penneri* | ATCC 33519 |
| *Bacillus pumilus* | ATCC 7061 | *Proteus vulgaris* | ATCC 9484 |
| *Bacillus subtilis* | ATCC 6633 | *Pseudomonas fluorescens* | ATCC 13525 |
| *Bacillus thuringiensis* | ATCC 10792 | *Pseudomonas fragi* | ATCC 4973 |
| *Carnobacterium divergens* | ATCC 35677 | *Pseudomonas syringae* | ATCC 19310 |
| *Citrobacter freundii* | ATCC 8090 | *Providencia rettgeri* | ATCC 29944 |
| *Clostridium botulinum* | ATCC 19397 | *Providencia stuartii* | ATCC 29914 |
| *Clostridium perfringens* | ATCC 10543 | *Serratia liquefaciens* | ATCC 12926 |
| *Enterobacter aerogenes* | ATCC 13048 | *Serratia marcescens* | ATCC 274 |
| *Enterobacter cloacae* | ATCC 13047 | *Stenotrophomonas maltophilia* | ATCC 13637 |
| *Enterobacter sakazakii* | ATCC 29544 | *Shewanella algae* | ATCC 51192 |
| *Hafnia alvei* | ATCC 9760 | *Shewanella baltica* | CECT 323 |
| *Klebsiella oxytoca* | ATCC 13182 | *Shewanella putrefaciens* | ATCC 8071 |
| *Raoultella planticola* | ATCC 33531 | *Staphylococcus aureus* | ATCC 9144 |
| *Klebsiella pneumoniae* | ATCC 10031 | *Staphylococcus aureus* | ATCC 35845 |
| *Listeria innocua* | ATCC 33090 | *Staphylococcus epidermidis* | ATCC 35983 |
| *Listeria ivanovii* | ATCC 19119 | *Staphylococcus xylosus* | ATCC 29971 |
| *Listeria monocytogenes* | CECT 4032 | *Vibrio alginolyticus* | ATCC 17749 |
| *Listeria seeligeri* | ATCC 35967 | *Vibrio parahaemolyticus* | ATCC 17802 |
| *Listeria welshimeri* | ATCC 35897 | *Vibrio vulnificus* | ATCC 27562 |
| *Morganella morganii* | ATCC 8076 | | |

products together with the mass lists of the reference strains present in the library. The agglomerative clustering method starts with creating one cluster for every peak list and calculates distances between the clusters. The two closest clusters, in this study the two clusters with the smallest average of pair wise distances (average linkage method), are then merged to a new cluster and distances are recalculated. This process is continued until only one single cluster remains. Distances between two peak lists were calculated, adding all individual similarity scores for every pair of two peak lists. Resulting distances varies between 1 for completely different set of peak masses, and zero for a perfect match. The width in peak match score was set to 5 Da.

## Results and Discussion

The aim of the present work was the application of MALDI-TOF MS fingerprinting for the identification of bacterial strains isolated from fresh and processed seafood samples. MALDI-TOF MS has shown to be a competent tool for the rapid and accurate differentiation of bacterial species, due to the resulting highly specific spectral profiles, named fingerprints (Giebel et al. 2010). For bacterial identification, the spectral profile of an unknown strain is compared to spectra of reference strains in a spectral library. In previous studies a wide library including mass spectral data of reference strains was constructed (Böhme et al. 2010; Fernández-No et al. 2010). The species used for the creation of the library are listed in Table 2 and included the main pathogenic and spoilage bacterial species potentially present in seafood. For this study 26 bacterial strains previously isolated from fresh fish and heat processed seafood (Table 1) were analyzed by MALDI-TOF MS and compared to the reference strains. For spectral analysis and the classification of the unknown strains, three approaches were carried out. First, spectral profiles were compared visually to the profiles of the reference strains. Although, most strains could be easily grouped on genus level by visual comparison, this approach is not applicable for species identification on a large scale. Thus, in a second approach, peak mass lists were extracted and compared to the peak mass lists of the reference strains, determining common peak masses with the web application SPECLUST. By this way, all studied strains could be identified on species level (Table 1), having most peak masses in common with the corresponding reference species. Finally, in a third approach, a dendrogram was constructed with SPECLUST by clustering peak mass lists of the isolated strains together with the peak mass lists of all reference strains. Clustering has proved to be a very competent method for bacterial classification and relations between the spectral profiles of the analyzed strains can be obtained more easily by grouping of the strains than by comparison of the spectral profiles visually or by searching common peak masses.

In Figs. 1, 2, and 3, spectral profiles of some strains isolated from seafood samples together with some reference strains are shown. The strains identified as *Pseudomonas* spp., *Proteus vulgaris*, *Serratia marcescens*, and *Stenotrophomonas maltophilia* could be easily classified visually on genus level, due to the high similarity of the spectral profiles to the corresponding reference spectra of these genera. Furthermore, when comparing the peak masses, a number of peaks could be found in common with the corresponding reference species and genus (inverted triangles in Figs. 1 and 2). In addition, the spectra of the *P. vulgaris* strains were also similar to the spectra of a *P. vulgaris* strain present in the database created by Mazzeo et al. (2006) and a number of peak masses could be found in common.

In the case of *Bacillus* spp. strains, the characterization on genus level was more difficult by visual comparison to reference spectra, due to that spectral profiles did not possess high similarities to any of the reference spectra. However, when searching for common peak masses most peak masses were found in common with the reference spectra of *B. cereus* and *Bacillus subtilis*, respectively (inverted triangles in Fig. 3).

In this sense, it has to be mentioned that the created spectral library of reference strains constituted a basis for the classification of unknown strains isolated from real seafood samples. However, strains isolated from nature, in this case from seafood samples, can vary significantly in their phenotypic and genotypic characteristics from the strains of culture collections, due to the different conditions of growth and the possible selective pressure. In our studies we observed that strains isolated from seafood samples, belonging to the same species, exhibited spectral profiles more similar to each other than to the spectra of the corresponding reference strain obtained from culture collections. Spectral variability on strain level has also been described by other authors (Bernardo et al. 2002; Donohue et al. 2006) and such mass variations may come from amino acid modifications of the same protein. Nevertheless, beside spectral variability within a species, some peak masses could be found in common for all strains of the same species (Table 3). Such characteristic peak masses that are common for a species as well as for a genus could serve as biomarker masses for the identification of the corresponding species and/or genus. However, the determination of specific peak masses is limited to the studied strains, but cannot give any assertion about the presence or absence of those biomarkers in bacterial species that were not considered in the performed studies. In this sense, in order to determine specific peaks and achieve a better identification of an unknown strain, a wide number of strains and species considered as reference would improve the accuracy of the method.

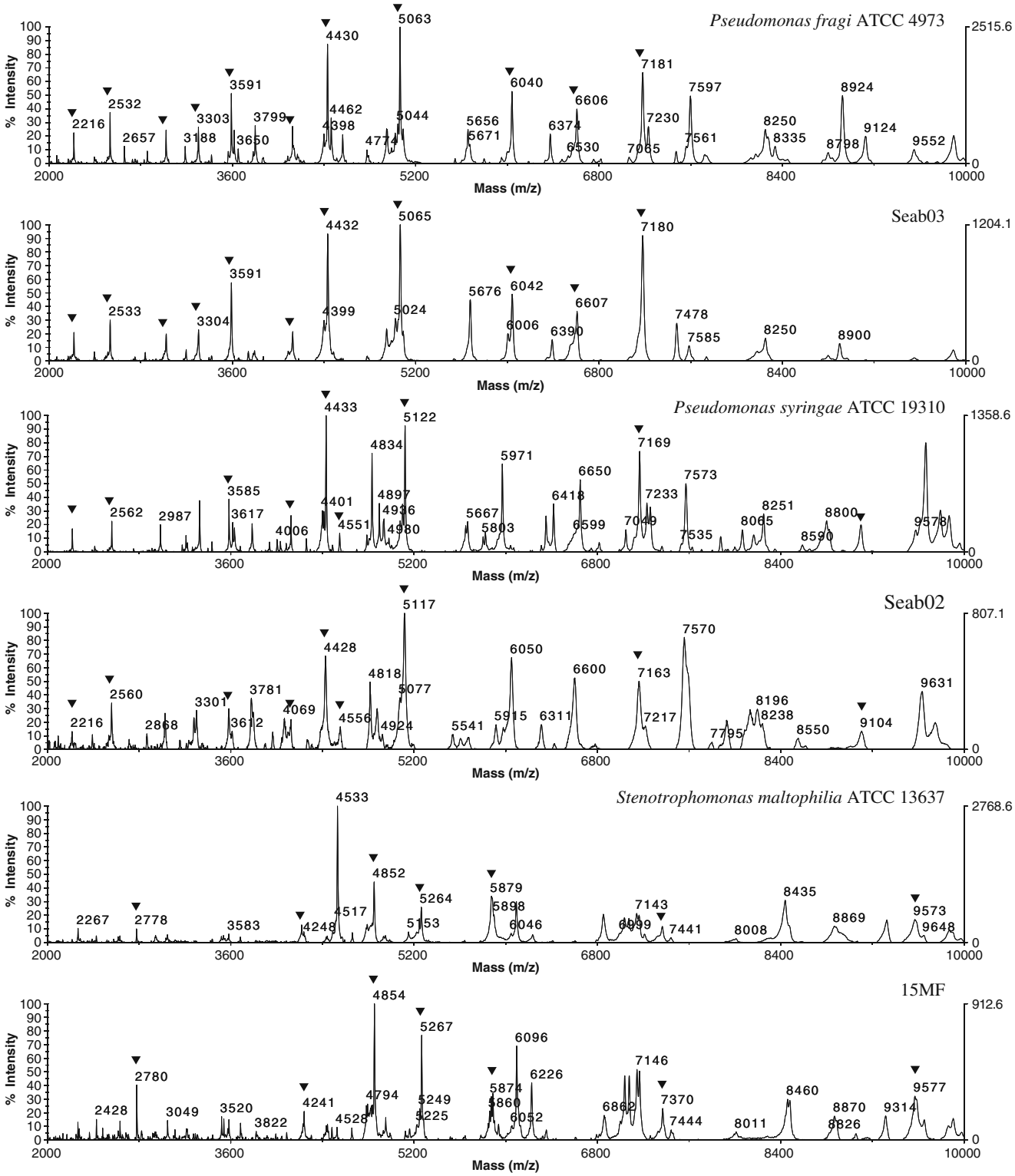


**Fig. 1** MALDI-TOF MS spectra of the reference strains *P. fragi* (ATCC 4973), *P. syringae* (ATCC 19310), *S. maltophilia* (ATCC 13637), and three strains isolated from seafood samples (Seab02, Seab03, and 15MF)

The clustering option of the web-based application SPECLUST is a rapid technique to analyze spectral profiles and to visualize spectral relations by grouping the obtained peak mass lists of all studied strains. Clustering of mass spectral data has been applied for the phyloproteomic study of different species and strains of the same species, with the aim to classify and differentiate the strains (Conway et al. 2001; Siegrist et al. 2007; Teramoto et al. 2007). In the

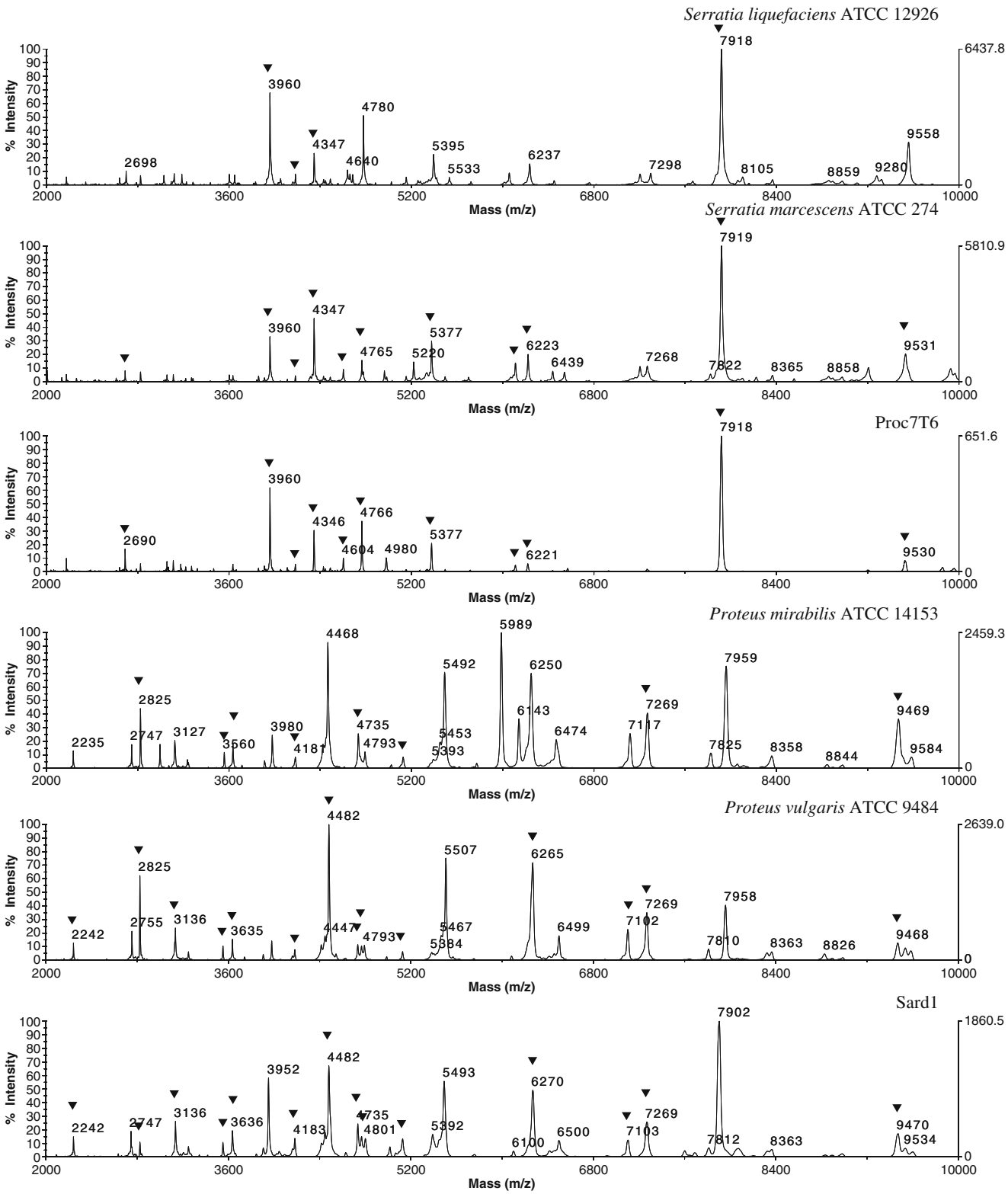


**Fig. 2** MALDI-TOF MS spectra of the reference strains *S. liquefaciens* (ATCC 12926), *S. marcescens* (ATCC 274), *P. mirabilis* (ATCC 14153), *P. vulgaris* (ATCC 9484), and two strains isolated from seafood samples (Sard1 and Proc7T6)

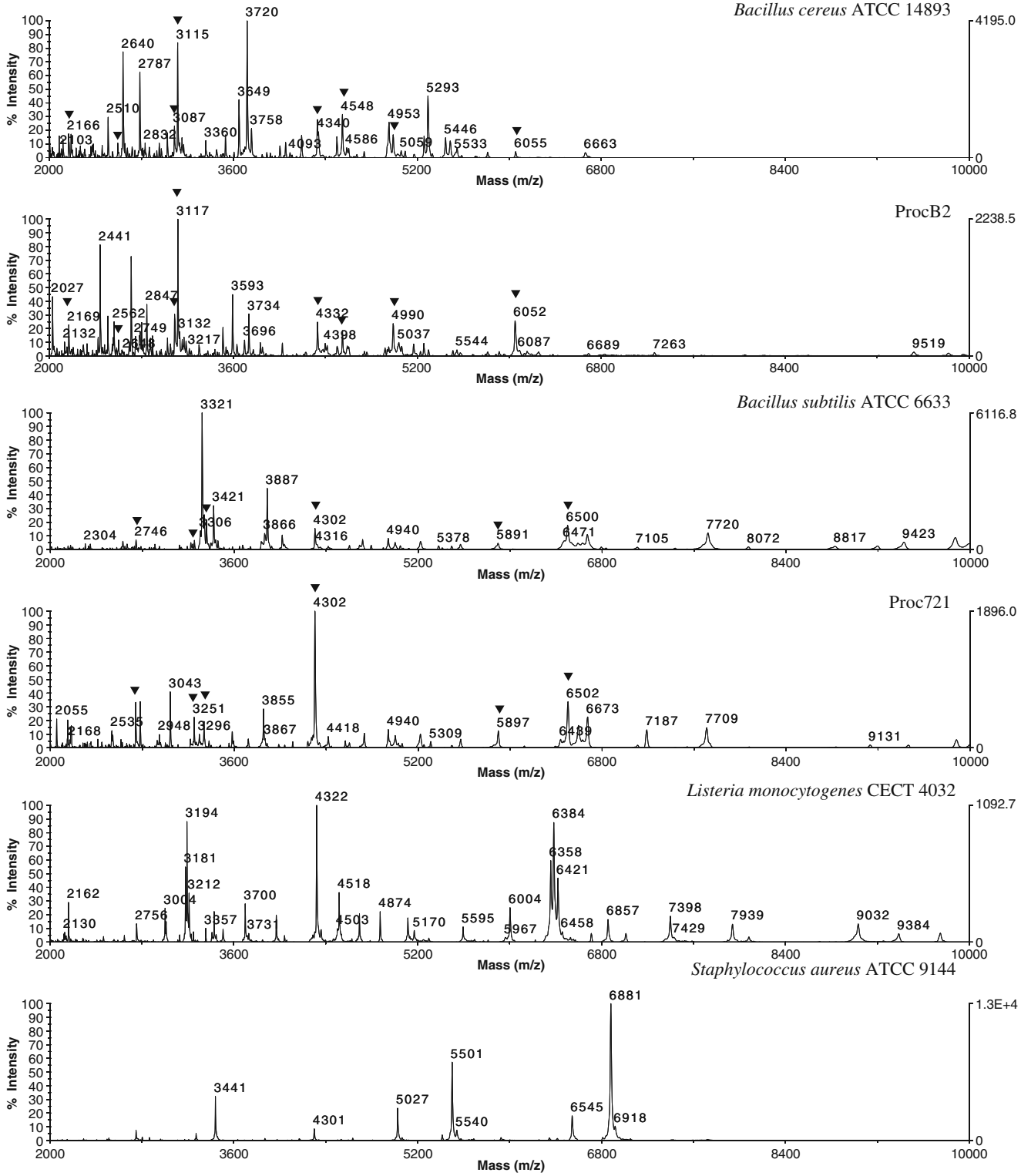


**Fig. 3** MALDI-TOF MS spectra of the reference strains *B. cereus* (ATCC 14893), *B. subtilis* (ATCC 6633), *L. monocytogenes* (CECT 4032), *S. aureus* (ATCC 9144), and two strains isolated from seafood samples (ProcB2 and Proc721)

present study, we applied the clustering technique for the classification of unknown strains isolated from fresh and processed seafood samples. Thus, aiming the identification of these strains a dendrogram was constructed by clustering peak mass lists of the strains isolated from seafood samples with the peak mass lists of the reference strains present in the library. In Fig. 4, an example of the cluster analysis is shown. It can be observed that the strains Sard1, Sard2,

**Table 3** Common peak masses for the strains isolated from seafood with the corresponding reference species or genus

| Bacterial species | No. of strains | Peak masses in common with the corresponding reference | |
|---|---|---|---|
| | | Species | Genus |
| A | | | |
| *Proteus vulgaris* | 4 | 4,768, 6,267 | 2,242, 2,824, 3,131, 3,552, 3,635, 4,182, 4,482, 4,735, 4,768, 5,128, 7,103, 7,269, 9,470 |
| *Pseudomonas fragi* | 5 | 3,022, 3,304, 3,592, 3,800, 6,041, 6,607, 7,181 | 2,217, 2,533, 4,126, 4,431, 5,064 |
| *Pseudomonas fluorescens* | 4 | 3,040, 3,408, 4,976, 6,076, 6,388, 9,054 | 2,217, 2,533, 3,584, 4,125, 4,431, 5,063, 7,166 |
| *Pseudomonas syringae* | 1 | 2,562, 4,551, 5,122, 9,099 | 2,218, 3,586, 4,126, 4,433, 7,169 |
| *Serratia marcescens* | 1 | 2,690, 4,604, 4,764, 5,376, 6,112, 6,221, 9,530 | 3,960, 4,182, 4,347, 7,918 |
| *Stenotrophomonas maltophilia* | 4 | 2,778, 4,238, 4,852, 5,264, 5,881, 7,367, 9,573 | – |
| *Bacillus cereus* | 2 | 2,167, 2,188, 2,594 | 3,087, 3,114, 4,329, 4,546, 4,989 |
| *Bacillus subtilis* | 5 | 2,745, 3,338 | 3,253, 4,302, 5,892, 6,500 |

Peak masses are presented as $[M+H]^+$ values

Sard3, and Sard4b were grouped together with the genus *Proteus* and were closer to *P. vulgaris* than to other species. The strain Proc7T6 was easily identified as *S. marcescens* due to the grouping with the reference strain ATCC 274. Likewise, the strains 15MF, 25MC6, Seab06, and Seab08 were grouped together with the reference strain *S. maltophilia* ATCC 13637 (Fig. 4). In the same way, all studied strains isolated from seafood samples showed a clear grouping together with the corresponding reference species and genus.

The identification by MALDI-TOF MS fingerprinting resulted that most of the bacterial strains isolated from fresh seafood were *Pseudomonas* spp. and *S. maltophilia* (Table 1). These species can be present in the indigenous microbiota of seafood and are adapted to cold temperatures (psychrotrophs), favoring their growth on products that are stored on ice (Elotmani et al. 2004; Gram and Dalgaard 2002). Remarkably, some strains isolated from fresh fish were identified as *P. vulgaris* that is a mesophilic bacterial species, growing best in moderate temperature. Bacterial strains isolated from fresh fish, were selected by their proteolytic character and their ability to produce histamine in the medium of Niven (Niven et al. 1981). All the identified species have been reported as weak histamine formers in seafood (Kim et al. 2003; Tsai et al. 2007) and the presence of these bacterial species in fish and other seafood products implies the risk of accumulating toxic levels of histamine and the subsequent negative consequences in consumer health. In addition, the species *S. maltophilia* is also a strong cadaverine producer and has been reported as an emerging pathogen that has been associated with a broad and increasing number of clinical syndromes (Ben-Gigirey et al. 2000).

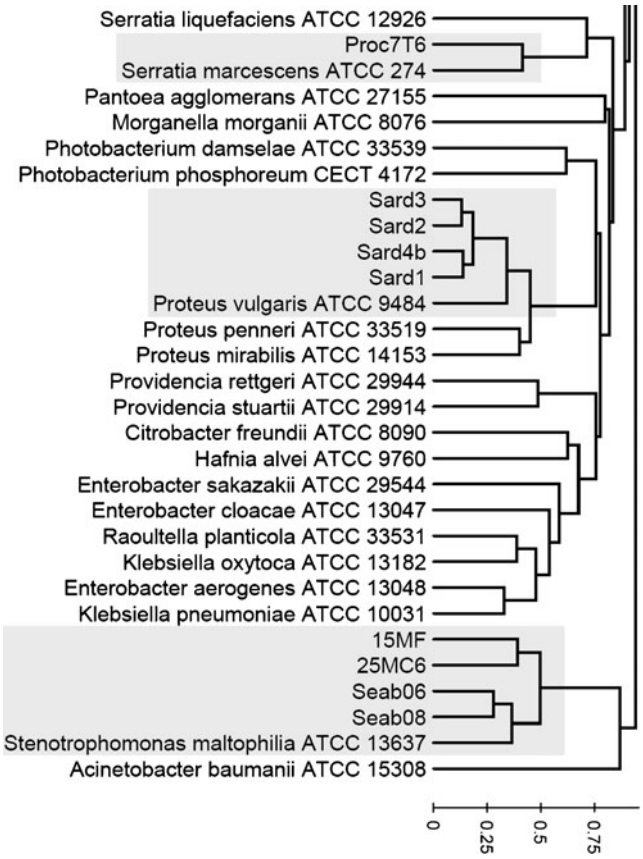


**Fig. 4** Dendrogram resulting from the cluster analysis of the peak mass lists of strains isolated from seafood and reference strains. The scale below the dendrogram indicates the relative distance between peak mass lists and varies between 1 for completely different set of peak masses and zero for a perfect match (see Materials and Methods)

On the other hand, strains isolated from processed, vacuum-packed seafood products that were subjected to a mild heat treatment, were identified mainly as *Bacillus* spp. (Table 1). In general, heat treatment of food products aims the inactivation of viable bacteria and thus the minimization of the risk of foodborne intoxications. However, some bacteria are able to survive light heat treatments, and spores of spore-forming bacteria, such as *Clostridium* spp. and *Bacillus* spp., are not destroyed by a pasteurization process. During the following processing and storage, spores can germinate and bacteria may grow. The isolation of *B. cereus* indicated a contamination of the corresponding seafood product, which can cause serious foodborne intoxications, when the product is stored under inappropriate temperature conditions. In addition, some strains were identified as *B. subtilis* that plays an important role in food spoilage. Furthermore, one strain was identified as *S. marcescens* that is a non-spore-forming bacteria. The survival of this strain during the heat treatment may be because this species can grow at higher temperature as 45 °C. Nevertheless, the survival of this species indicated an insufficient heat treatment process.

The identification of the isolated strains by MALDI-TOF MS fingerprinting concurred with the results obtained by the genomic approach. However, it should be mentioned that, when applying genomic tools, some strains could not be identified on species level. Thus, in the case of the genera *Pseudomonas* and *Bacillus*, species identification was not always possible by sequencing the 16S rRNA gene, due to the high similarity of sequences of various species of the same genus. It has already been reported that 16S rRNA gene sequences of *B. cereus* and *Bacillus thuringiensis* do not allow differentiation of these species (Daffonchio et al. 2006). Similarly occurs in the case of the species *Pseudomonas fragi*, *Pseudomonas fluorescens*, and *Pseudomonas syringae*. In contrast, the analysis by MALDI-TOF MS exhibited spectral differences, allowing the classification of *Pseudomonas* strains as well as *Bacillus* strains into different groups. In the case of the reference strains *B. cereus* and *B. thuringiensis*, although their spectra had many peak masses in common, some spectral differences could be observed. Thus, strains isolated from seafood samples that could not be differentiated between *B. cereus* and *B. thuringiensis* by 16S rRNA gene analysis, were identified as *B. cereus* by MALDI-TOF MS, because they had more peak masses in common with the reference spectrum of this species. As a conclusion, for some bacterial species, the analysis by MALDI-TOF MS resulted to be more discriminating than the analysis based on genomic tools. A higher discrimination potential of MALDI-TOF MS fingerprinting in comparison to genomic tools was also reported by other authors (Vargha et al. 2006).

Thus, MALDI-TOF MS analysis can be successfully applied to the identification of species on seafood, either fresh or processed, being a fast and accurate method, since the identification may be achieved in a short time after isolation compared to other methods that take longer time. Furthermore, the described method can be applied to any other foodstuffs, since the constituted spectral library may easily be enlarged by further bacterial species and strains that are of interest in the corresponding food product. In this sense, MALDI-TOF mass fingerprinting proved to be an accurate and cost-effective technique, with potential for routine identification of bacteria in the food sector as well as in clinical microbiology (Seng et al. 2009). In this sense, some authors consider that, taking into account the cost of materials and staff, the cost of bacterial identification by MALDI-TOF MS fingerprinting is around two-thirds less than conventional methods (Hsieh et al. 2008; Nassif 2009). On the other hand, it has several advantages over other fast methods relying on genomics, such as DNA microarrays, because fewer steps are necessary to achieve bacterial identification and thus, fewer errors are introduced along the analyzing process. Another advantage of MALDI-TOF mass fingerprinting is the effortless analysis of results, since no extensive data processing and statistical analysis is required, as it is the case in other rapid methods for bacterial identification, such as FT-IR and DNA microarrays.

## Conclusions

The strains isolated from fresh fish and processed seafood products included important foodborne pathogens that can cause serious foodborne intoxications. Furthermore, strains that are capable to decarboxylate histidine were isolated from fresh fish, which could imply a health risk for the consumers due to the possible elevated levels of histamine. In addition, several isolated strains were identified as particular spoilage species that could cause a rapid deterioration of the products of marine origin and result in high economic losses.

Pathogenic and spoilage bacterial species detection and identification is an important issue for the seafood industry, where a rapid assessment of a potential microbial risk can avoid high economic losses and health hazards by the application of preventive measures, being aimed at the identified bacterial risk. The results of this study showed that MALDI-TOF MS fingerprinting is a feasible, rapid, and cost-effective technique for the classification and identification of unknown bacterial strains isolated from fresh and processed seafood samples. The vast spectral data can be effectively examined by searching common peaks masses and cluster analysis. In further studies, a large number of samples with more different species should be taken into consideration, together with the analysis of sample mixtures. In order to shorten the time, the

automatization of the spectral analysis would be desirable. Future work will include the publication of the created spectral library, including strains obtained from culture collections, as well as strains isolated from seafood samples, to allow other researchers the comparison of their spectra to the library and thus, a more accurate and rapid identification of bacteria in seafood.

**Acknowledgments** The authors thank Dr. Francisco Barros (Unidad de Medicina Molecular, Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela) for his excellent technical assistance with 16S rRNA sequencing. This work was funded by the PGIDIT Research Program (Project PGIDIT06PXIB261164PR) of the Xunta de Galicia (Galician Council for Industry Commerce and Innovation). The work of K. Bohme and I.C. Fernandez-No is supported by a "Maria Barbeito" and "Lucas Labrada" research contract from Xunta de Galicia.

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