MALDI-TOF MS for the identification and characterization of *Flavobacterium* isolates from water and the environment

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Abstract

Flavobacteriosis, in Switzerland caused mainly by *Flavobacterium psychrophilum* (Flavobaceriaceae), has become an important problem for the Swiss fish farming industry. While records of skin and gill infections have been described since a long time, systemic infections, known as the so-called rainbow trout fry syndrome (RTFS), have been reported for the first time not until 1992. Since then, occurrence of RTFS has been observed regularly in Swiss fish farms, at yearly frequencies of 4.3–12.9%.

The taxonomy and nomenclature of Flavobacterium species is in continuous change. Presently 16S rRNA gene PCR is used for taxonomic purposes and, in outbreaks, it serves as a complement to classical microscopy for the identification of pathogenic species, but this technique is relatively expensive and time consuming. Matrix-Assisted Laser-Desorption/Ionisation Time-Of-Flight mass spectrometry (MALDI-TOF MS) is now increasingly used in clinical diagnostics to identify bacteria, fungi and other microorganisms [1,2]. MALDI-TOF MS is mainly targeting ribosomal proteins and has proven to be extremely specific and very reliable [3]. The potential of MALDI-TOF MS in diagnosis of diseases of aquatic organisms has not yet been explored.

There is a need for a fast, reliable and cost-effective identification technique targeting in particular pathogenic species of Flavobacteria. The aim of this study was to test the suitability of MALDI-TOF MS for the identification of *Flavobacterium* species, in particular the pathogenic species *F. psychrophilum, F. columnare* and *F. branchiophilum*. Own strains of *F. branchiophilum, F. columnare, F. johnsoniae, F. aquatile, F. fryxellicola, F. frigidimaris, F psychrolimnae,* and *Chryseobacterium* sp., as well as type strains of *F. psychrophilum* (DSM3660), *F. succinicans* (DSM4002), *F. aquidurense* (DSM18293), *F. limicola* (DSM15094), *F. pectinovorum* (DSM6368), *F. hydatis* (DSM2063), and *F. hercynium* (DSM18292) were used to build up a reference database.

The set of test samples consisted of a total of 130 isolates collected from water (n=30), fish tissue (*n*=92) and tank swabs (*n*=8) from Swiss fish farm environments. The samples were collected during 2009 and 2010. DNA of all samples was extracted with the Instagene kit (Bio-Rad, Hercules, CA, USA). 16S rRNA gene sequencing was performed using the universal primers uniL 26f (5'-ATTCTAGAGTTTGATCATGGCTCA-3') and uniR 1392r (5'-ATGGTACCGTGTGACGGGCGGTGTGTA-3') [4].

For MALDI-TOF MS pure cultures were grown on CY-Agar for 5 days at 15°C. All samples were processed with a MALDI-TOF MS Axima Confidence™ spectrometer (Shimadzu-Biotech Corp., Kyoto, Japan) in linear mode (m/z = 2000–20,000). A loop (1 µl) of bacteria was mixed with 10 µl of a 5% formic acid solution and incubated for 5 min. Then, 10 µl of a sinapinic acid matrix (Matrix substance for MALDI-MS, Fluka, Switzerland) was added to the solution. Analyses were run in duplicate by spotting 2 µl of each suspension in FlexiMass™ target wells and letting them air dry at room temperature. The reference strain *Escherichia coli* K12 (GM48 genotype) was used as standard for calibration and reference for quality control. Protein mass profiles were obtained and the spectral analysis was carried out using the software package SARAMIS™ (BioMérieux, Switzerland) at default settings.

Phylogenetic trees (neighbor joining analysis) were constructed using sequences of the 16S rRNA gene with the software MEGA, version 4 [5] and compared with dendrograms of the spectral profiles obtained by MALDI-TOF-MS. Agglomeration algorithm was single-linkage clustering using the Dice similarity coefficients (SARAMIS™, error 0.08%, 3,000–20,000 m/z). Protein profile analysis by MALDI-TOF MS yielded species-specific spectra for the pathogenic species *F. psychrophilum*, *F. columnare* and *F. branchiophilum* as well as for other species (Fig. 1).

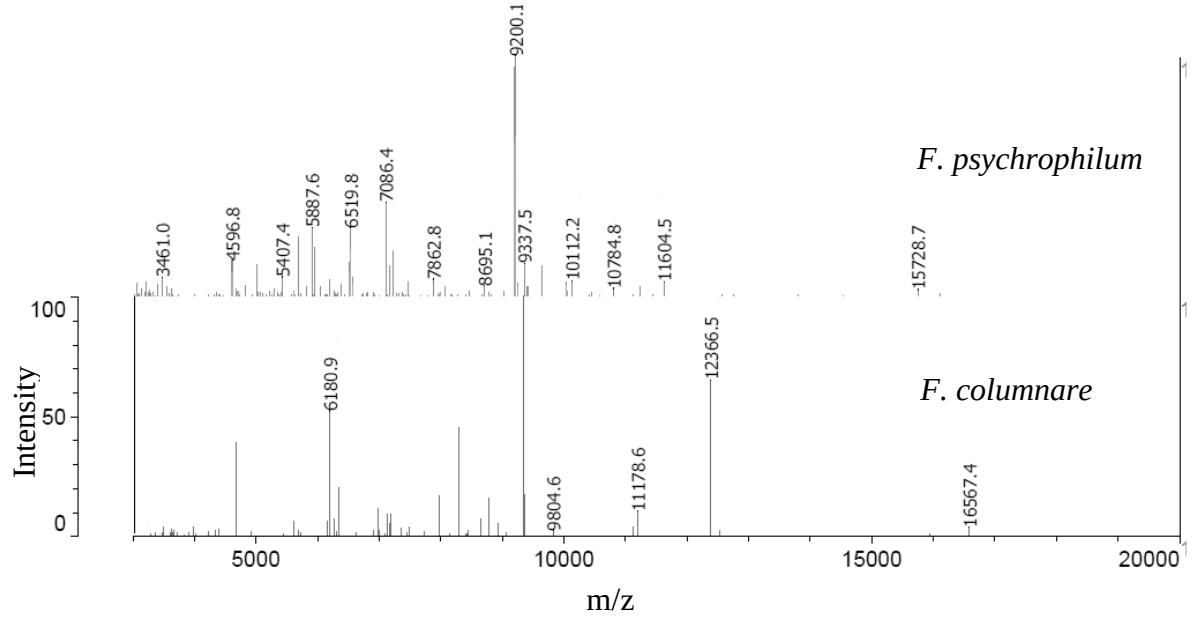

Figure 1. Protein spectra profiles of *F. psychrophilum* and *F. columnare*. Proteins are separated by their mass-to-charge ratio.

Of the total 130 field samples examined in this study, 126 were identified by means of 16S RNA gene sequencing as belonging to *Flavobacterium* spp., while 4 samples belonged to *Chryseobacterium* spp. MALDI-TOF MS correctly identified the 126 samples of *Flavobacterium* spp. at the genus level and distinguished them unequivocally from the 4 *Chryseobacterium* isolates. Thus, the generic sensitivity and specificity of MALDI-TOF MS was 100%.

Clustering of Flavobacterium species by phenetic analysis of the MALDI-TOF data and by phylogeny of 16S rRNA gene was almost identical. This is not surprising, because ribosomal proteins which were utilized in the MALDI-TOF analysis – directly and closely reflect the 16S RNA structure.

At the species level, MALDI-TOF-MS identified reliably all *F. psychrophilum* (*n*=61), *F. hercinium* (*n*=7), *F. succinicans* (*n*=5), *F. frigidimaris* (*n*=2), *F. aquatile* (*n*=1), *F. columnare* (*n*=1), and *F. psychrolimnae* (*n*=1). Only 5 environmental samples of *F. aquidurense* could not be identified by MALDI-TOF MS (Tab. 1).

Table 1. *Flavobacterium* species and number of samples per each species identified by MALDI-TOF MS and 16S rRNA sequencing. Percent of agreement between the two techniques is listed in the last column.

Species	MALDI-TOF	16S rRNA	Agreement
F. aquatile	1	1	100%
F. columnare	1	1	100%
F. psychrolimnae	1	1	100%
F. frigidimaris	2	2	100%
F. succinicans	5	5	100%
F. hercynium	7	7	100%
F. psychrophilum	61	61	100%
F. aquidurense	0	5	-

All samples of the pathogenic species *F. psychrophilum* and *F. columnare* were correctly identified by MALDI-TOF MS. In several studies of clinically relevant, human and animal pathogenic bacteria and fungi the identification efficiency of MALDI-TOF MS was shown to be high and the technique very rapid and cost-effective [1,6]. MALDI-TOF MS costs are very reduced (Approximately € 1–2, as compared to € 20 for 16S rRNA gene analysis). After isolation in pure cultures, strains can be identified within few minutes, as compared to an almost two days' work needed for 16S rRNA gene analysis.

In our study, only *F. aquidurense* could not be conclusively identified by MALDI-TOF MS, failure to identify this cluster could have been due to 16S rRNA low resolution. In fact, other studies have recently shown that this gene is not always useful to resolve phylogenies. For *Aeromonas* spp., for instance, 16s rRNA sequences produced a cluster that might actually be composed by different species [7–8].

In conclusion MALDI-TOF MS allowed a rapid and cost-effective identification of *Flavobacterium* isolates and the results reflected the taxonomy derived from 16S rRNA gene sequencing. Inclusion of well-characterized samples in the MALDI-TOF database used, however, is an important pre-requisite for a reliable identification of taxonomically inhomogeneous species.

A limitation of MALDI-TOF MS resides in the need of pure cultures before identification can be done. Recent studies with clinically relevant pathogens, on the other hand, have shown that a direct identification of microorganisms in infected samples (e.g. blood cultures) is possible [9]. Work is currently underway to evaluate the usefulness of MALDI-TOF MS to directly detect *F. psychrophilum* in infected fish tissue and also to detect mixed infections in fish tissue or in cultures.

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