Establishment and Application of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for Detection of Shewanella Genus

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Shewanella species are widely distributed in the aquatic environment and aquatic organisms. They are opportunistic human pathogens with increasing clinical infections reported in recent years. However, there is a lack of a rapid and accurate method to identify Shewanella species. We evaluated here matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for rapid identification of Shewanella. A peptide mass reference spectra (PMRS) database was constructed for the type strains of 36 Shewanella species. The main spectrum projection (MSP) cluster dendrogram showed that the type strains of Shewanella species can be effectively distinguished according to the different MS fingerprinting. The PMRS database was validated using 125 Shewanella test strains isolated from various sources and periods; 92.8% (n = 116) of the strains were correctly identified at the species level, compared with the results of multilocus sequence analysis (MLSA), which was previously shown to be a method for identifying Shewanella at the species level. The misidentified strains (n = 9) by MALDI-TOF MS involved five species of two groups, i.e., Shewanella algae–Shewanella chilikensis–Shewanella indica and Shewanella seohaensis–Shewanella xiamenensis. We then identified and defined species-specific biomarker peaks of the 36 species using the type strains and validated these selected biomarkers using 125 test strains. Our study demonstrated that MALDI-TOF MS was a reliable and powerful tool for the rapid identification of Shewanella strains at the species level.

Keywords: MALDI-TOF mass spectrometry, detection, Shewanella, multilocus sequence analysis, establishment and application
INTRODUCTION

The genus *Shewanella* comprises a group of oxidase-, catalase-, and ornithine decarboxylase-positive and H₂S-producing, facultative anaerobic bacteria with a wide distribution in the environment. It plays an important ecological role in many fields such as materials engineering, environmental engineering (Zou et al., 2018), and marine biology (Gorby et al., 2006; Fredrickson et al., 2008; Kouzuma et al., 2015; Daefller et al., 2017). However, *Shewanella* is a common source of food spoilage bacteria, in particular seafood; and *Shewanella* contamination of foods during food processing and storage adversely affects the production, transportation, and sales (Hau and Granick, 2007; McLean et al., 2008; Wang et al., 2009; Janda and Abbott, 2014). *Shewanella* is also an opportunistic pathogen of humans (Erfanmanesh et al., 2019). Through occupational or recreational activities, exposure to the marine environment containing *Shewanella* or ingestion of marine organisms contaminated by *Shewanella* (Janda and Abbott, 2014) may cause a range of infections including skin and soft tissue infections (SSTIs), invasive diseases, hepatobiliary diseases, otitis media and associated sequelae, and other infections (Janda and Abbott, 2014). Some studies have shown that SSTI is the most common clinical features of *Shewanella* infection, including cellulitis, abscess, or necrotizing fasciitis (Yousfi et al., 2017). In recent years, *Shewanella* has been isolated from more and more clinical specimens (Janda and Abbott, 2014).

The genus *Shewanella* has a high diversity with more than 70 species reported so far⁴. An efficient and accurate method for the identification of *Shewanella* species is still lacking. Traditional methods are mainly based on phenotypic tests combined with biochemical identification, which is time-consuming, laborious, and even inaccurate. The widely used 16S rRNA gene as bacterial species identification tool (Yarza et al., 2014) has been found to lack the power to identify *Shewanella* at the species level (Sun et al., 2013; Glaeser and Kämpfer, 2015). The housekeeping gene *gyrB* was found to have a higher resolution than 16S rRNA for *Shewanella* species identification (Bozal et al., 2002; Miyazaki et al., 2006; Sung et al., 2012), but no standardized cutoff value has been established for the identification (Bozal et al., 2002; Miyazaki et al., 2006; Sung et al., 2012). We previously established that the method of multilocus sequence analysis (MLSA) can accurately identify *Shewanella* at the species level (Fang et al., 2019). This method requires PCR and sequencing of six housekeeping genes from each isolate, which is time-consuming and costly. Thus, the method is unsuitable for clinical diagnostic laboratories.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a powerful technology for rapid microbial identification in recent years. It has a short turnaround time for species identification to enable targeted treatment. The principle of MALDI-TOF MS based on species identification is that each species has its characteristic MS spectra that provide a good resolution for differentiation of bacteria at the species level (van Belkum et al., 2015). MALDI-TOF MS has been rapidly developed and widely used in clinical laboratories (Angeletti, 2017; Schubert and Kostrzewa, 2017), due to its advantages of convenient sample preparation, simple experimental operation, and high identification accuracy (Samantha et al., 2018). A key requirement of MALDI-TOF MS based on species identification is a specific and accurate spectra database (Jang and Kim, 2018), which must be established for the targeted species.

The objectives of this study were to establish a peptide mass reference spectra (PMRS) database of *Shewanella* species by MALDI-TOF MS and to validate the effectiveness of MALDI-TOF MS and the database for identifying *Shewanella* species, using type strains of 36 species and 125 test strains derived from clinical, environmental, and food samples.

### MATERIALS AND METHODS

#### Bacterial Strains and Cultivation Methods

A total of 161 *Shewanella* strains were used in this study including 36 type strains and 125 test strains. All type strains were sourced from the China General Microbiological Culture Collection Center (CGMCC), the German Collection of Microorganisms and Cell Cultures [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)], the Japan Collection of Microorganisms (JCM), the Korean Collection for Type Cultures (KCTC), the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG Bacteria Collection), and the Marine Culture Collection of China (MCCC). Detailed information of type strains is listed in Table 1. The three non-*Shewanella* strains, namely, *Vibrio cholerae* N16961, *Pseudomonas aeruginosa* 09MAS0023, and *Aeromonas hydrophila* 2247, were sourced from Center for Human Pathogenic Culture Collection (CHPC). The test strains were isolated from different sources in China, during years 2007–2020, including clinical (n = 75), food (n = 39), and environmental (n = 11) isolates. The 36 *Shewanella* type strains were incubated on marine agar 2216E at suitable temperatures according to the protocols provided by each pathogen culture collection center. The test strains were identified by API20E (bioMérieux SA) according to instrument of manufactory and incubated at 37°C for 18–24 h for subsequent identifications. *Escherichia coli* ATCC 25922 was used for the calibration of the instrument.

#### Sample Preparation for Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS

An ethanol/formic acid method was used for protein extraction (Anja and Sascha, 2009). One loop of fresh bacterial culture was thoroughly suspended in 300 µl of ultrapure water, and then 900 µl of absolute ethanol was added. The mixtures were centrifuged at 14,000 × g for 5 min. Then the supernatant was discarded, and the pellet was allowed to dry at room temperature. Next, equal volumes of 70% formic acid and acetonitrile were added to the pellet in sequence. After the pellet was well suspended, it was centrifuged at 14,000 × g

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⁴http://www.bacterio.net/shewanella.html
for 3 min, and the whole cell extracts were transferred to a clean tube. The matrix solution consisted of acetonitrile (500 μl), ultrapure water (475 μl), trifluoroacetic acid (25 μl), and supersaturated α-cyano-4-hydroxycinnamic acid (CHCA) for the identification at the species level, and log scores < 2.0 and ≥ 1.7 were taken as the identification at the genus level or the presumptive species level identification. Log scores below 1.7 were considered unreliable.

Each sample was coated with 12 targets. At least 24 high-quality spectra with stable baseline, abundant protein peaks and even distribution were selected. The software of iDBac (version 1.1.10) was used to create the dendrogram based on the main

### Parameter Setting, Spectrum Generation, and Identification

Mass spectrometry analysis was performed using MALDI-TOF MS EXS3000 (Zybio Inc., Chongqing, China). MS spectra were obtained in linear mode within a range of 2,000–20,000 Da. E. coli ATCC 25922 was used for mass calibration and instrument parameter optimization, to make the average deviation of molecular weight less than 300 ppm after correction. MS data were analyzed by MDT Master (version 1.1). As specified by the manufacturer’s instructions, log scores ≥ 2.0 were accepted for the identification at the species level, and log scores < 2.0 and ≥ 1.7 were taken as the identification at the genus level or the presumptive species level identification. Log scores below 1.7 were considered unreliable.

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### Table 1 | Detailed information of 36 Shewanella type strains.

| No. | Species               | Strain     | Isolation place            | Isolation source                  | Isolation year|
|-----|-----------------------|------------|----------------------------|-----------------------------------|---------------|
| 1   | Shewanella aestuarii  | JCM 17801T | Suncheon Bay, Korea        | A tidal flat                      | 2011          |
| 2   | Shewanella algicola   | KCTC 2359T | Jeju Island, Korea         | Brown algae, Sargassum thunbergii | 1995          |
| 3   | Shewanella algaea     | DSM 9439   | Japan                      | Oil brine                         | 1998          |
| 4   | Shewanella baltica    | KCTC 22121T| Jeju Island, Korea         | Marine black sand                 | 2008          |
| 5   | Shewanella carassii   | MCCC 1A00763 | Anhui, China               | Surface of crucian carp, Carassius carassius | 1997          |
| 6   | Shewanella chilikensis| JCM 21332T | Orissa, India              | Sediment of a lagoon              | 2007          |
| 7   | Shewanella decolorationis | JCM 21555 | Guangzhou, China          | Activated sludge                  | 2002          |
| 8   | Shewanella electrodiphila | KCTC 22431T | Korea                      | Deep-sea sediment                 | 2007          |
| 9   | Shewanella gaetbuli   | DSM 6066   | Arctic Ocean               | Marine sediment                   | 2001          |
| 10  | Shewanella indicus    | JCM 23171T | Kanwar jetty, India        | Sediment of the Arabian Sea       | 2006          |
| 11  | Shewanella invenionis | JCM 24955T | Okinawa Trough             | Deep-sea sediment                 | 2014          |
| 12  | Shewanella kairetica  | DSM 19170T | Suruga Bay, Japan          | Deep-sea sediment                 | 2007          |
| 13  | Shewanella livingstonensis | KCTC 23961T | Saemankum, Korea          | A tidal flat sediment             | 2008          |
| 14  | Shewanella mangrove   | LMG 23744T | Baltic Sea, Denmark        | Cod                               | 2002          |
| 15  | Shewanella paetitulism | KCTC 22180T | Baltic Sea, Denmark        | Cod                               | 2001          |
| 16  | Shewanella pacifica   | JCM 11558T | Yokohama, Japan            | Squid body                        | 1994          |
| 17  | Shewanella profunda   | JCM 12192  | Yellow Sea, Korea          | Sea water                         | 2001          |
| 18  | Shewanella putrefaciens| JCM 2080T  | Pacific Ocean              | Deep marine sediment              | 2011          |
| 19  | Shewanella salinae    | ATCC 8071T | England                    | Butter                            | 1931          |
| 20  | Shewanella saea        | JCM 190105T| Pacific Ocean              | Saury intestine                    | 1995          |
| 21  | Shewanella schlegeliana| KCTC 23568T| Saemankum, Korea          | A tidal flat sediment             | 1997          |
| 22  | Shewanella vesiculosa  | DSM 2142T | Deception Island           | Marine sediments                  | 1998          |
| 23  | Shewanella xiamenensis| MCCC 1A00763 | Fujian, China             | Coastal sea sediment              | 1998          |

# "-" means isolation year unknown.
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FIGURE 1 | Dendrogram of the cluster analysis of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra. The scale below the dendrogram represents the degree of difference in the mass spectrometry fingerprinting of the 36 Shewanella type strains, and the difference level reflects the relationship between each other, with the value between 0 and 1. With the main spectrum projection (MSP) similarity of 77.6% as the critical value, 36 type strains of Shewanella were divided into six groups. Among them, Algae, Gelidii, Aquimaria, and Putrefaciens included multiple type strains (species); S. aestuarii and S. hanedai stood separately in their own clade.

spectrum projection (MSP), using the algorithm of unweighted pair-group method with arithmetic means (UPGMA). Three non-Shewanella strains were included as controls.

Multilocus Sequence Analysis of Shewanella Test Strains
Genomic DNAs were extracted according to the standardized instructions of the DNA extraction kit (TaKaRa, Dalian, China). Six single-copy housekeeping genes (gyrA, gyrB, infB, recN, rpoA, and topA) were selected according to previous studies (Fang et al., 2019). Housekeeping genes of the 36 type strains were obtained from GenBank (Supplementary Table 1). DNAStar software was used to join the upstream and downstream sequences. MEGA 6.06 was used to compare the concatenated sequences and build the neighbor-joining phylogenetic tree. In terms of parameter setting of the evolutionary tree, Kimura’s two-parameter model with the pairwise-deletion option was used. The robustness of tree topologies was evaluated with 1,000 bootstrap replications, and values > 70% were shown at the nodes of the branches.

Analysis of Specific Biomarker Peaks in Mass Spectrometry
Mass spectrometry analysis was performed using the MDT Master software to calculate the height and area of spectrum peaks, and Welch’s t test was used to determine peaks with statistical differences. Finally, an output file was generated. The peaks with relative signal intensity greater than 2% were extracted, and normality test (W test) was performed on the distribution of the peaks. Next, Kruskal–Wallis H test (when P-value < 0.1 in the W test) was used to test pairwise difference of the peak distribution between strains. In all cases, P-value < 0.05 was considered significant. The spectra peaks from different type strains were collected together, and non-redundant candidate species-specific biomarker peaks were computed for the 36 Shewanella species using MDT Master software.
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RESULTS

Construction of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS Database for Shewanella Genus

In this study, the PMRS database containing 36 type strains of different Shewanella species was constructed. About 100 peaks were detected in the MS fingerprinting of each strain, where a large majority of the peaks were concentrated in the range of 3,000–10,000 m/z. The dendrogram based on MSP of 36 Shewanella showed long terminal branches that separate species from the nearest counterparts (Figure 1), and the variance of peaks distribution between branches ranged from 33.9% (S. marinintestina–S. pneumatophore) to 84.5% (S. aestuarii). As the only genus in the family of Shewanellaceae, there was large difference in spectra between Shewanella and its closely related genera, which were considered as outgroups. No misidentification was observed at the genus level. The 36 type strains of different Shewanella species were divided into six clades, using the 77.6% difference in MSP as the cutoff value. The clade of Algae, Gelidii, Aquimarina, and Putrefaciens included multiple species, while S. aestuarii and S. hanedai were well separated as single species clades from the other clades.

Species Identification of Test Strains by Multilocus Sequence Analysis

Multilocus sequence analysis was used to provide a reference identification of all test strains. The concatenated sequences data were used to construct a phylogenetic tree (Figure 2). The test strains were clustered with type strains for unambiguous species identification. Among the 125 strains, 83 (66.4%) were S. algae, 15 (12.0%) S. chilikensis, 13 (10.4%) S. indica, 9 (7.2%) S. xiamenensis, 3 (2.4%) S. seohaensis, and 2 (1.6%) S. carassii. Except for S. seohaensis with all isolates from environmental specimens, the other five species contained isolates from clinical samples.

Species Identification of Test Strains by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS

Under the condition of signal-to-noise ratio >3.0, approximately 100 peaks were detected in the range of 2,000–20,000 m/z. When identifying the test strains, online comparison searches were performed against an expanded database (Zybio Inc., Chongqing, China, containing the Shewanella PMRS database). The final MALDI-TOF MS identification results for the 125 test strains were S. algae (n = 87), followed by S. chilikensis (n = 14), S. indica (n = 10), S. xiamenensis (n = 8), S. seohaensis (n = 4), and S. carassii (n = 2).

Comparison of Results Between Multilocus Sequence Analysis and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS

Since MLSA has been confirmed to be accurate for identifying Shewanella at the species level (Fang et al., 2019), all 125 test strains were analyzed by MLSA, and the results were used to evaluate the effectiveness of MALDI-TOF MS for species identification. Taking the MLSA identification results as the “true species identity” of a test strain, all strains were identified correctly at the genus level, and 116 (92.8%) of the test strains were accurately identified at the species level. Nine strains were misidentified by MALDI-TOF MS, involving five...
species of two groups, i.e., *S. algae*–*S. chilikensis*–*S. indica* and *S. seohaensis*–*S. xiamenensis*. More specifically, one *S. algae* strain was misidentified as *S. chilikensis*, while the two *S. chilikensis* strains and the three *S. indica* strains were wrongly identified as *S. algae*; two strains of *S. xiamenensis* were misidentified as *S. seohaensis*, and one strain of *S. seohaensis* was wrongly identified as *S. xiamenensis* (Figure 3).

**Analysis of Potential Species-Specific Biomarker Peaks**

Due to the misidentification of nine strains by MALDI-TOF MS, we analyzed the spectra data of the type strains to determine whether there are potential species-specific biomarker peaks that can be used to improve the accuracy. The output file of peak intensity for different type strains was generated by MDT Master Software. After the mass spectrum peaks with relative intensity < 2% were removed, the remaining peaks were collected for further analysis. Owing to the non-normal data, $P < 0.05$ (Kruskal–Wallis $H$ test) was considered significant in peak distribution. Biomarker peaks were detected using peak lists among 36 type strains (Table 2), and 125 test strains were examined for verification. The potential species-specific biomarker peaks were located within the range of 2,000–12,000 m/z. The nine wrongly identified strains were correctly identified at the species level by using the biomarker peaks. An average of eight species-specific peaks was found for each type strain, while *S. aestuarii* JCM 17801 harbored 21 specific peaks, higher than the average amount. Furthermore, *S. aestuarii*, *S. aquimarina*, *S. baltica*, *S. carassii*, and *S. gaetbuli* type strains harbored multiple specific peaks in a relative-low range (2,000–3,000 m/z).

In the comparison of MS fingerprinting among *S. algae*–*S. chilikensis*–*S. indica*, the specific peaks were 10,065 m/z (*S. algae*), 3,307 m/z (*S. chilikensis*), and 4,827 m/z (*S. indica*) (Figure 4A). Similarly, for the *S. xiamenensis*–*S. seohaensis* group, the peak with 4,221 m/z only appeared in *S. xiamenensis*, while peaks of 3,778 m/z and 9,574 m/z were present in *S. seohaensis* (Figure 4B).

**DISCUSSION**

Multiple *Shewanella* species are frequently isolated from food products and clinical specimens as opportunistic pathogens (Pagani et al., 2003; Liu et al., 2013; Janda and Abbott, 2014). Commercial systems, such as Vitek and MALDI-TOF MS (bioMérieux, Bruker), are available for species identification in clinical laboratories (Regoui et al., 2020; Sánchez-Juanes et al., 2020). However, only a few species, like *S. putrefaciens* and *S. algae*, were recorded in the database (Liu et al., 2013; Janda and Abbott, 2014; Zhang et al., 2018). Several other *Shewanella* species have often been misidentified by biochemical tests or MALDI-TOF MS (Byun et al., 2017; Zhang et al., 2018). Therefore, we urgently need an expanded database to identify *Shewanella* species correctly.

MALDI-TOF MS has revolutionized the routine identification of microorganisms in clinical laboratories by introducing a simple, rapid, high-throughput, and low-cost technology (O’Connor et al., 2016; Bao et al., 2018). The two key requirements of MALDI-TOF MS microbial identification are the species coverage in the database and the representativeness of bacterial diversity used for the database construction (Rodriguez-Temporal et al., 2017; Honnavar et al., 2018; Paul et al., 2019).
In this study, the PMRS database included the type strains of 36 Shewanella species for MALDI-TOF MS identification at both genus and species levels. The MSP dendrogram was used to evaluate the distance and relationship of the type strains that represent the 36 Shewanella species. The tree topology confirmed that each species is well separated. The most recent common ancestor in the MLSA phylogenetic tree was divided into two groups, Algae, Gelidii, Aquimarina, and Putrefaciens, were also grouped together respectively as expected, confirming the spectra contained sufficient signal for species identification. The identification accuracy of the MALDI-TOF MS was validated using 125 test strains, the species identity of which were confirmed by MLSA. The 125 test strains analyzed belonged to six different species, namely, S. algae, S. chilikensis, S. indica, S. xiamenensis, S. soehaensis, and S. carassii. Thus, we can confidently conclude that the MALDI-TOF MS method developed here is capable of identifying Shewanella species. Although there are over 70 Shewanella species and other species remain to be tested, these six species are the most commonly isolated species from clinical samples, and other species are relatively rare (Zhang et al., 2018). The testing of other 30 Shewanella species included in this database and additional species not included in this study will further expand the utility of this method.

However, in our study, nine out of 125 strains were misidentified. These nine isolates belonged to five species, which were divided into two groups, S. algae–S. chilikensis–S. indica and S. xiamenensis–S. soehaensis. The strains of each group shared the main peaks represented by ribosomal proteins in the spectrum.
for strain identification (Ryzhov and Fenselau, 2001; Bremer and Dennis, 2008; Nakamura et al., 2016). It is known to be difficult to distinguish by MALDI-TOF MS Shigella spp. from E. coli (Wieser et al., 2012), Brucella melitensis from Ochrobactrum anthropi (Poonawala et al., 2018), and some species within the genus Bacillus (McLaughlin et al., 2014), as there is very little difference between them in the spectra of ribosomal proteins.

In order to overcome the limitations of MALDI-TOF MS in differentiating closely related species, potential species-specific biomarker peaks were found to be useful. Peaks in the range of 2,000–20,000 m/z are more likely to be ribosomal proteins that are discriminatory at the species level. Ha et al. (2019) successfully applied low-mass profiling to identify species-specific mass peaks for the identification of two genetically closely related Bacillus species. In this study, we took a similar approach and identified species-associated biomarker peaks based on the type strains, validated using the 125 test strains. With the use of the species biomarker peaks, nine misidentified test strains can be accurately identified at the species level. We identified 2–21 species-associated biomarkers for the species level identification. However, it should be noted that these species-associated biomarkers were identified based on one type strain; and thus when more strains of a given species are included, some of these markers may become variable within a species or non-species specific. For the species with multiple test strains available, the species-associated biomarker appears to be species specific.

CONCLUSION

The establishment of the PMRS library provides the technical basis for the detection and identification of Shewanella species that are relevant to food safety and clinical disease. The study revealed that MALDI-TOF MS could be a fast and relatively inexpensive method for the identification of the Shewanella genus. Species-specific biomarker peaks were identified and employed to improve the identifications at the species level. MALDI-TOF MS can effectively replace traditional identification methods for the identification of Shewanella.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DW designed the work. KY and ZH performed the experiments. KY, ZH, YL, QF, LL, SW, HD, HC, and YX collected the samples and isolated strains. KY, ZH, and RL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.625821/full#supplementary-material
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Conflict of Interest: QF, LL, and SW were employed by the company Zybio Inc. Chongqing, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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