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Estimates of abundance and diversity of *Shewanella* genus in natural and engineered aqueous environments with newly designed primers

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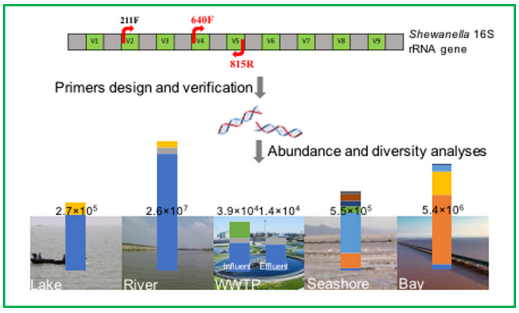
HIGHLIGHTS

- New *Shewanella* primers exhibited a high coverage and specificity.
- More diverse *Shewanella* exist in freshwater habitats than previous reports.
- *Shewanella* distribution in freshwater is greatly different from that in marine systems.
- Hydrolyzation tank in anaerobic/oxic process in WWTPs is a habitat for *Shewanella*.
- *Shewanella* prefers to thrive in nutrient abundant environments.

GRAPHICAL ABSTRACT



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ABSTRACT

Shewanella species have a diverse respiratory ability and wide distribution in environments and play an important role in bioremediation and the biogeochemical cycles of elements. Primers with more accuracy and broader coverage are required with consideration of the increasing number of *Shewanella* species and evaluation of their roles in various environments. In this work, a new primer set of 640F/815R was developed to quantify the abundance of *Shewanella* species in natural and engineered environments. *In silico* tools for primer evaluation, quantitative polymerase chain reaction (qPCR) and clone library results showed that 640F/815R had a higher specificity and coverage than the previous primers in quantitative analysis of *Shewanella*. Another newly developed primer pair of 211F/815cR was also adopted to analyze the *Shewanella* diversity and demonstrated to be the best candidate in terms of specificity and coverage. We detected more *Shewanella*-related species in freshwater environments and found them to be substantially different from those in marine environments. Abundance and diversity of *Shewanella* species in wastewater treatment plants were largely affected by the process and operating conditions. Overall, this study suggests that investigations of abundance and diversity of *Shewanella* in various environments are of great importance to evaluate their ecophysiology and potential ecological roles.

1. Introduction

Since the first discovery in food spoilage in 1931, an increasing number of *Shewanella* species have been isolated and identified (Derby and Hammer, 1931; Hau and Gralnick, 2007). *Shewanella* species are known for their diverse respiratory ability, being capable of reducing various

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electron acceptors such as iron oxide, manganese oxide, dimethyl sulfoxide, some radionuclides and toxic elements like Cr (Hau and Gralnick, 2007; Middleton et al., 2003; Nealson and Saffarini, 1994; Nealson and Scott, 2006; Xie et al., 2018). They have been found in a wide range of environments and play an important role in biogeochemical cycles of multiple elements, bioremediation of radionuclides and toxic elements, and bioenergy production processes (Brauer et al., 2011; Hau and Gralnick, 2007; Sani et al., 2008; Sun et al., 2016; Venkidusamy et al., 2016). However, due to the lack of suitable primers for *Shewanella* detection, the abundance and diversity of the *Shewanella* species in environments, especially in freshwater ecosystems, are not well known.

Several primers, including a 16S ribosomal RNA (rRNA)-targeted oligonucleotide probe SPN3 and a primer set of Sw.783-F/Sw.1245-R, have been developed to detect *Shewanella* species in freshwater sediments (DiChristina and DeLong, 1993; Snoeyenbos-West et al., 2000). In a later study, two primer sets, 1492R/SPN3 and She211F/She1259R, were designed sequentially to detect *Shewanella* genus in a minerotrophic wetland, but only two species were detected (Todorova and Costello, 2006). Primer set She120F/220R was furthermore developed to quantify *Shewanella* species in a sand cap and 10^4–10^6 gene copies per milliliter porewater were detected (Himmelheber et al., 2009). This primer set was later used to detect *Shewanella* in subsurface sediments and *Shewanella* was observed sporadically (Lin et al., 2012). *Shewanella* species or other species were detected only intermittently with the existing primers (Kim et al., 2012; Lin et al., 2012; Lu et al., 2010; Snoeyenbos-West et al., 2000). Several studies have shown that diverse *Shewanella* species were isolated and detected from wastewater treatment plants (WWTPs) and freshwater lakes and that they showed great potential in bioremediation (Li et al., 2014; Liu et al., 2016; Tomczyk-Zak et al., 2013; Xu et al., 2005; Ye and Zhang, 2013). Our previous work also revealed that a large of exoelectrogenic bacteria exist in fresh lake sediments and WWTPs (Yang et al., 2016). We therefore hypothesized that members of the *Shewanella* genus, known exoelectrogenic bacteria, may have wider distribution in freshwater than reported previously. Current primer sets may underestimate the number of *Shewanella* species in freshwater systems and further investigations with better tools should be conducted.

In this work, *Shewanella*-specific primers 640F and 815R were designed for a quantitative polymerase chain reaction (qPCR) analysis based on the alignment of *Shewanella* 16S rRNA gene sequences. Their specificity and coverage were compared with the previous primers to analyze the same samples collected from freshwater environments. Samples from marine environments were also used to make a comparison. In addition, the feasibility of using the newly designed primers for *Shewanella* species diversity detection in different aqueous environmental samples was also explored, especially in WWTPs. In this way, the abundance and diversity of *Shewanella* genus in diverse natural and engineered aqueous environments were evaluated.

2. Materials and methods

2.1. Primer design and validation

In order to design new *Shewanella*-specific primers, >1000 *Shewanella* 16S rRNA gene sequences with >1000 base pairs were retrieved from Genbank databases and then aligned using clustalx2.1 alignment program (Larkin et al., 2007). Conserved regions in the hypervariable regions of *Shewanella* 16S rRNA gene were identified and used as the targets for degenerate primers design. The primer 640F in the V4 hypervariable region and primer 815R in the V5 hypervariable region as qPCR primers were designed based on the alignment. The sequences of the primer set 640F/815R are shown in Table 1. The specificity and coverage of all previous primers and new primers were tested *in silico* using TestPrime program against SILVA SSU r126 database (https://www.arb-silva.de/search/testprime) (Quast et al., 2013) and ProbeMatch tool against the RDP's database (http://rdp.cme.msu.edu/probematch/search.jsp) (Cole et al., 2014). 61 nearly full-length 16S rRNA gene sequences representing 61 different *Shewanella* species were also downloaded from both databases to construct the phylogenetic tree. Primers for qPCR were also tested based on the 61 sequences by using the ProbeMatch tool of RDP.

2.2. Sites descriptions and sampling

To test the specificity and coverage of the newly designed primers and investigate the distribution characteristics of *Shewanella*, diverse environmental samples were collected from freshwater lake sediments, river sediments, wetland soil, WWTPs, an intertidal zone of sea and ocean sediments. The locations of the freshwater lake sediments, river sediments, wetland soil and two WWTPs are shown in Fig. S1. The ocean sediment sample was collected from Hangzhou Bay (HB, 121°35′E and 30°30′N), which is not indicated on the map, in China. The samples collected at the intertidal zone (ZS) of the East China Sea were gifted by Prof. Baolan Hu at Zhejiang University, China (Wang et al., 2017). The lake sediment was sampled from Chaohu Lake (CL, 117°16′ - 117°51′ E and 31°43′ - 31°25′ N), one of the five largest freshwater lakes in China with severe eutrophication (Chen et al., 2011). The river sediment was sampled from Nanfei River (NR) in Hefei, China, which is heavily polluted (Qian et al., 2011). Wetland soil samples were collected from a creek shore in a wetland park (WP) near Chaohu Lake.

The wastewater samples were collected from the three municipal WWTPs operated with different processes in China, *i.e.*, Wangtang wastewater treatment plant (WW) with an oxidation ditch process, Zhuzhuanjing wastewater treatment plant (ZW) with a sequencing batch reactor (SBR) process, and Bengbu wastewater treatment plant (BW) with an anaerobic/oxic process. We collected duplicate samples from each treatment step at each WWTP. Specifically, in WW, total fourteen samples were collected from the influent, anaerobic tank, anoxic zone in the oxidation ditch, aerobic zone in the oxidation ditch, recycling sludge, sludge thickening tank, and secondary settling tank. In ZW, total six samples were collected from the influent, SBR, and effluent. In BW, total eight samples were collected from the anoxic tank, aerobic tank, hydrolysis tank, and effluent. These samples were stored in −20 °C for chemical and molecular analysis.

2.3. DNA extraction and PCR amplification

Genomic DNA was extracted from the environmental samples using the Power Soil DNA Kit (Mo Bio Laboratories, USA). All steps were conducted according to the manufacturer's instructions. The integrity of the extracted DNA was checked with gel electrophoresis. DNA concentration and quality were determined by a Nanodrop spectrophotometer (Thermo Scientific Inc., USA). The designed primer sets 640F/815R and 120F/815cR and previous primer sets were used for the specific PCR amplification of the *Shewanella* 16S rRNA gene. PCR amplification for each replicate of each sample was performed in a 25-μl solution containing 2.5 μl of PCR 10× buffer (Takara Co., Japan), 2 μl of dNTP mixture (2.5 mM each), 0.125 μl of Ex Taq DNA polymerase, 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 1 μl of template DNA (10 ng/μl), and Milli-Q water. The sequences of the primers and PCR conditions are listed in Table 1. The thermal profiles were optimized based on the melting temperature (Tm) and the amplified fragment size of primers by parallel PCR with different annealing temperatures.

2.4. Quantitative PCR analysis

Quantitative PCR analysis was performed on a StepOne real-time PCR instrument (Applied Biosystems Inc., USA) with SYBR Green qPCR kit (Takara Co., Japan). The final concentration of each primer was 400 nM and the thermal profiles are shown in Table 1. The quantitative