Journal of Microbiological Methods 86 (2011) 62–68

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A rapid fingerprinting approach to distinguish between closely related strains of *Shewanella*

Jinjun Kan [a,*,1], Beverly Flood [b,1], John P. McCrow [b], Ji S. Kim [b], Lynette Tan [b], Kenneth H. Nealson [a]

[a] Department of Earth Sciences, University of Southern California, Los Angeles, CA 90089, USA
[b] Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA

ARTICLE INFO

Article history:
Received 10 March 2011
Received in revised form 28 March 2011
Accepted 28 March 2011
Available online 6 April 2011

Keywords:
Shewanella
ITS–DGGE
Bacterial isolation
MFCs

ABSTRACT

One of the big operational problems facing laboratories today is the ability to rapidly distinguish between strains of bacteria that, while physiologically distinct, are nearly impossible to separate based on 16S rRNA gene sequence differences. Here we demonstrate that ITS–DGGE provides a convenient approach to distinguishing between closely related strains of *Shewanella*, some of which were impossible to separate and identify by 16 rRNA gene sequence alone. Examined *Shewanella* genomes contain 8–11 copies of rrn (ribosomal RNA gene) operons, and variable size and sequence of 16S–23S ITS (intergenic transcribed spacer) regions which result in distinct ITS–DGGE profiles. Phylogenetic constructions based on ITS are congruent with the genomic trees generated from concatenated core genes as well as with those based on conserved indels, suggesting that ITS patterns appear to be linked with evolutionary lineages and physiology. In addition, three new *Shewanella* strains (MFC 2, MFC 6, and MFC 14) were isolated from microbial fuel cells enriched from wastewater sludge and identified by ITS–DGGE. Subsequent physiological and electrochemical studies of the three isolates confirmed that each strain is phenotypically/genotypically distinct. Thus, this study validates ITS–DGGE as a quick fingerprint approach to identifying and distinguishing between closely related but novel *Shewanella* ecotypes.

Published by Elsevier B.V.

1. Introduction

The genus *Shewanella* is a ubiquitously distributed and environmentally important clade, especially at redox interfaces (reviewed in Hau and Gralnick, 2007; Fredrickson et al., 2008). This gammaproteobacterium is a facultative anaerobe that has been isolated from both free-living and symbiotic systems with a wide range of salinities, temperatures, and barometric pressures (Hau and Gralnick, 2007). *Shewanellae* have been extensively studied for their ability to use a broad range of electron acceptors for respiration and as such, *Shewanellae* are currently used in a number of biotechnological applications to include bioremediation of radionuclides and heavy metals and more recently in microbial fuel cells (MFCs) (Bretschger et al., 2007; Hau and Gralnick, 2007; Fredrickson et al., 2008).

Despite the quite variable genotypic and phenotypic traits, *Shewanellae* are a distinct phylogenetic clade. One challenge working with closely related bacterial strains is confirming the identity of laboratory stocks and experimental cultures. Mislabeling and/or cross-contamination can easily occur when working with closely related strains. Therefore, it is critical to implement a simple, rapid and reliable technique to routinely monitor stock cultures and experiments. However, simply sequencing the 16S rRNA gene (Amann et al., 1995) may not provide an unambiguous answer, as at the strain level, it may lack the resolution required to separate closely related strains. Bacterial ribosomal RNA genes are typically positioned in ribosomal RNA operon (s) along the genome with the order of 16S, 23S, and 5S. The region between the small (16S) and big (23S) ribosomal RNA genes is called intergenic transcribed spacer (ITS). The size and sequences of ITS are often variable within multiple rrn operons of the same organism (Dolzani et al., 1994; García-Martínez et al., 1999; Tannock et al., 1999), and as such have been used in typing and identifying diverse groups of bacteria (Gurtler and Stanisich, 1996; Jensen et al., 1993; Chun et al., 1999; Maeda et al., 2000; Shaver et al., 2001; Gonzalez-Escalona et al., 2007). Additionally, the variability in the number of rrn operons between more distally related strains also imparts distinct profiles amenable to DGGE–ITS for microbial community fingerprinting (Fisher and Triplett, 1999; Brown et al., 2005).

DGGE (denaturing gradient gel electrophoresis) separates DNA amplicons by both size and GC content, and has a resolution of up to one base pair difference (Myers et al., 1985; Sheffield et al., 1989). It is a quick approach and has been widely used to characterize and monitor variations of microbial communities (Muyzer et al., 1993; Heuer et al., 1997). The combination of ITS and DGGE yields distinct band patterns and therefore provides better resolution for discriminating closely-related phylotypes. In fact, ITS–DGGE has been applied to genotyping

* Corresponding author at: Microbiology Division, Stroud Water Research Center, Avondale, PA 19311, USA. Tel.: +1 610 268 2153x280; fax: +1 610 268 0490.
E-mail address: jkan@stroudcenter.org (J. Kan).
[1] These authors contribute equally to the manuscript.

0167-7012/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.mimet.2011.03.017

members of *Escherichia*, *Lactobacilli*, *Pseudomonas*, *Rhizobium*, *Staphylococcus*, *Streptomyces*, and *Vibrio* (Buchan et al., 2001; D'Elia et al., 2007; Nour, 1998; Yang et al., 2000; de Oliveira et al., 1999; Mendoza et al., 1998; Park and Kilbane, 2006; Chun et al., 1999; Gonzalez-Escalona et al., 2007). However, little is known about the variability of the ITS region in *Shewanella* and the feasibility of using ITS to distinguish *Shewanella* species/strains.

As genomic information rapidly accumulates for *Shewanella*, sequences for the ITS regions have become publicly available. Currently, more than 20 genomes of genus *Shewanella* have been fully sequenced (www.ncbi.nlm.nih.gov). In this study, 15 *Shewanella* strains with known genomes were selected and fingerprinted using ITS–DGGE technique. The results demonstrate that compared to 16S rDNA-DGGE, ITS–DGGE yields better resolution and is capable of differentiating between very closely related *Shewanella* strains, including subspecies. As such, we employed this technique to aid in identifying three novel strains of *Shewanella* isolated from MFCs (Kan et al., 2011). Lastly, we confirmed that the isolates are unique ecotypes through characterizing carbon source utilization under aerobic conditions as well as power production in MFCs.

2. Materials and methods

2.1. Shewanella collections

Our laboratory maintains stocks of 15 *Shewanella* type strains, most of which have been sequenced. These included *Shewanella paleana*, *Shewanella loihica* PV4, *Shewanella woodyi*, *Shewanella denitrificans* OS217, *Shewanella frigidimarina* NCIMB400, *Shewanella baltica* OS155, *Shewanella baltica* OS195, *Shewanella* sp. W3-18-1, *Shewanella putrefaciens* CN-32, *Shewanella putrefaciens* 200, *Shewanella* sp. MR-4, *Shewanella* sp. ANA-3, *Shewanella* sp. MR-7, *Shewanella oneidensis* MR-1, and *Shewanella amazonensis* SB2B. Frozen stocks were streaked out on LB agar plates from which an isolated colony was used to inoculate LB broth overnight at 30 °C.

2.2. Bacterial isolation from microbial fuel cells

Both aerobic and anaerobic bacteria were isolated from MFCs fed with 6 different carbon sources at 5 mM: lactate, succinate, NAG (N-acetyl glucosamine), acetate, formate and uridine (Kan et al., 2011). Briefly, aliquots of microbial communities enriched at the anodes were streaked on minimal medium plates (1.5% agar) (Bretschger et al., 2007) with corresponding carbon sources from which they were enriched and incubated at room temperature under aerobic (3 days) and under anaerobic conditions with Fe-citrate provided as the electron acceptor (6 days). Colonies were picked from plates and transferred to respective liquid media for subsequent analysis. Isolates other than *Shewanella* were not discussed herein.

2.3. DNA extraction and PCR

Bacterial genomic DNA was obtained from 1 mL culture cell pellets by use of lysozyme, proteinase K, and SDS (sodium dodecyl sulfate) concomitant with phenol–chloroform extraction and isopropanol precipitation (Schmidt et al., 1991). After drying the DNA pellet with centriVap concentrator (LABCONCO), DNA was dissolved in PCR water and stored at 4 °C for future analysis. DNA concentrations were determined based on absorbance at 260 nm with a Spectrophotometer ND-1000 (Nanodrop). PCR amplification was performed in a 50 mL reaction containing approximately 25 ng of template DNA, 25 mL PCR Mastermix (Qiagen), 0.5 mM (each) primer, and PCR H_2O. Two pairs of common DGGE primers were used to amplify different regions of 16S rRNA gene: 1070f–1392r (Ferris et al., 1996) covers the V7–V8 region, while 341f–907r (Scäfer and Muyzer, 2001) amplifies the V3–V5 region. Primers for ITS–DGGE were 16S-1406f and 23S-66R (GC) (Suzuki et al., 2004). Reverse primer 23S-66R (GC) contained a 40 bp GC-rich clamp at the 5′ end (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG). The PCR program for 1070f and 1392r (GC) was as previously described (Kan et al., 2006). For both 341f–907r and 1406f-66R, the PCR programs followed the protocol described by Scäfer and Muyzer (2001). PCR amplification was performed with a Mastercycler gradient (Eppendorf). Agarose gel electrophoresis was used to detect and estimate the concentrations of PCR amplicons. Primers 27F and 1492R (Lane, 1991) were used to amplify and sequence the 16S rRNA gene for the isolated bacterial strains from MFCs by using Bigdye-terminator chemistry by ABI PRISM3100 Genetic Analyzer (Applied Biosystems). The sequence data from new isolated *Shewanella* has been deposited in GenBank with accession nos. HM589853–HM589855.

2.4. DGGE and gel image analysis

DGGE was performed as previously described (Muyzer et al., 1993; Kan et al., 2006) except with a linear gradient of the denaturants of 40–60%. The DGGE gel was stained with SYBR Gold and photographed (Øvreås et al., 1997) with a CCD camera mounted on a UV transluminator (UVP). Banding patterns (band positions, presence and absence of the bands, etc.) of ITS–DGGE were compared and analyzed to differentiate *Shewanella* strains. In order to test the reproducibility of 16S-DGGE and ITS–DGGE, independent DNA extractions and experiment setups were repeated at least 3 times.

2.5. Phylogenetic analyses

Small subunit ribosomal RNA gene sequences of 15 *Shewanella* spp. were retrieved and compared with the NCBI database using BLASTN. All the closest related sequences were aligned in the phylogenetic analysis, including the 39 type species reviewed by Hau and Gralnick (2007). Near complete gene sequences (>1400 bp) were used for the sequence alignment that was performed with CLUSTALW. Positions where gaps outnumbered characters were excluded and this resulted in an analysis of 1197 characters for *Shewanella* spp. The phylogenetic tree and bootstrapping of distance (Jukes–Cantor neighbor-joining) were calculated by MacVector 10.0 software package (GCG) based on 1000 resampling datasets. Phylogeny and bootstrapping of parsimony and maximum likelihood were performed by PAUP 4.0b10 for Macintosh program (Swofford, 1998) and PHYLIP 3.67 (Felsenstein, 2005) based on 100 resampling datasets, respectively.

2.5.1. Phylogeny constructions of ITS

The multiple copies of ITS sequences from each species were first divided among 4 general categories of homology. Representative ITS sequences from each species in each category were concatenated to generate a species tree based on potentially orthologous ITS sequences, and 100 random samples were taken. Each sample was aligned with CLUSTALW, and error-prone gaps were removed using GBLOCKS (Castresana, 2000) prior to phylogenetic inference on each sample and a final consensus tree built with PHYLIP (Felsenstein, 2005).

2.6. Physiology of newly isolated Shewanellae

Carbon source utilization was examined for the newly isolated *Shewanella* strains and closely related type strains by using the Biolog PM1 and PM2A MicroPlates™ system (Table 2). The conduct of the experiment was performed following the manufacturer's instructions (http://www.biolog.com/index.html). The PM1 and PM2A MicroPlates™ were incubated at room temperature and scanned after 24 h and 48 h using a FLUOstar OPTIMA (BMG Labtech). Weakly respired substrates yielded somewhat variable reactions with the tetrazolium