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Nitrate-reducing community in production water of three oil reservoirs and their responses to different carbon sources revealed by nitrate-reductase encoding gene (*napA*)

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ABSTRACT

The nitrate-reducing microbial community in oil reservoirs was examined by PCR using primers to amplify a segment of *napA* gene encoding for a subunit of the nitrate reductase, and the effects of different organic carbon additions on the nitrate-reducing community were also evaluated. The orders *Rhodocyclales* and *Burkholderiales* within *Betaproteobacteria* and *Pseudomonadales* within *Gammaproteobacteria* were recovered in production water of all three oil reservoirs. Amendment of organic acids promoted *Enterobacteriales* within *Gammaproteobacteria* and orders of *Rhodocyclales*, *Pseudomonadales*, and *Burkholderiales*, while alkanes favored the *Rhizobiaceae* family within *Alphaproteobacteria* and orders of *Rhodocyclales* and *Pseudomonadales*. Results indicated that the functional gene *napA* can be used as a valuable biomarker in analyzing the diversity of nitrate reducers in oil reservoirs. Nitrate-reducing microbial community shifts following the available carbon sources. Information about *napA* gene in oil reservoirs environment is scarce. This is the first study that combines molecular and culture-dependent approaches to reveal the diversity of the nitrate-reducing microbial community in production water of oil reservoirs by using the *napA* gene.

1. Introduction

Microbial denitrification is widely reported in soils (Gu et al., 2007; Wakelin et al., 2009), rumen, wastewater treatment, and activated sludge, and in wastewater (Chon et al., 2009), but hardly in production waters from oil reservoirs. Production water in the petroleum industry is wastewater produced during extraction of oil from oil reservoirs, and it contains crude oil and inorganic salts, including sulfate and chloride from the subsurface environment. Hydrogen sulfide (H_2S) produced by sulfate-reducing bacteria (SRB) in oil reservoirs is a main cause of corrosion and souring. To inhibit SRBs, nitrate injection is commonly used in water-flooded oil reservoirs, and it may change the microbial community in the subsurface environment by enriching nitrate-reducing bacteria (NRB) (Hubert and Voordouw, 2007; Schwermer et al., 2010).

Nitrate-reducing prokaryotes constitute a diverse group within the *Alpha*, *Beta*, and *Gammaproteobacteria*, gram-positive bacteria, and even Archea, sharing the common biochemical capability of obtaining energy from dissimilatory reduction of nitrate (Philippot et al., 2002; Baek et al., 2003; Li et al., 2011). Microbial denitrification, a respiratory process, consists of four consecutive reaction steps in which nitrate is reduced to dinitrogen (N_2) gas ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Zumft, 1997). The denitrification process involves four kinds of metalloenzymes: nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductases (Canfield, 2010). Nitrate reductases are widespread in bacteria and three types of nitrate-reducing systems, cytoplasmic nitrate reductase, membrane-bound respiratory nitrate reductase, and periplasmic nitrate reductase, have been reported (Richardson et al., 2001). Nitrate reductases can reduce NO_3^- to NO_2^- and be divided into NAR and NAP complexes. Denitrifying bacteria are reported to contain one or both of the nitrate reductases (Carter et al., 1995; Roussel et al., 2005). NAR complex is membrane-bound with the NarG catalytic protein located in the cytoplasm while NAP complex is equally membrane-bound but its NapA protein is periplasmic (Bru et al., 2007). NapA protein presents a dilemma as it can be involved in redox balancing, respiration, and NO_3^- uptake (Berks et al., 1995; Carter et al., 1995; Castillo et al., 1996; Flanagan et al., 1999). Polymerase chain reaction (PCR) amplification for *napA* genes in nitrate-reducing bacterial communities have been reported before (Flanagan et al., 1999; Philippot et al., 2002). A gene cluster encoding components of a putative

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periplasmic nitrate reductase system (*napAGHBFLD*) has been sequenced (Simon et al., 2003; Martinez-Espinosa et al., 2006; Klotz and Stein, 2008; Bazan et al., 2008). Also, previous research indicates analysis of partial *narG* genes resulted in poor resolution for environmental samples, but well-defined results have been obtained from the partial *napA* fragments (Alcantara-Hernandez, 2009). Therefore, *napA* genes rather than *narG* genes were used to provide a sound basis for assessing the taxonomic composition of nitrate-reducing communities in the oilfields of this study.

In this study, the objectives were to elucidate the diversity of the nitrate-reducing community in production water from three oil reservoirs using the *napA* functional gene as a biomarker and to evaluate the influences of organic carbon additions on the diversity of the nitrate-reducing microbial community.

2. Materials and methods

2.1. Sample collection

Samples of the production water were taken from three oil reservoirs with different temperatures—one from the Shengli oilfield, and two from the Huabei oilfield of P.R. China. The in-situ oil reservoir temperature was 60 °C and total salinity was about 8425–11,196 mg l^{-1} at the Zhan 3 unit of the Shengli oilfield, Shandong Province, P.R. China. The in-situ oil reservoir temperature and total salinity were 37 °C and 1301 mg l^{-1} at the Menggu Lin unit, and 58 °C and 6199 mg l^{-1} at the Ba 19 unit, both in the Huabei oilfield, Inner Mongolia, P.R. China. In total, 5 l of production water were taken from a central production well at each location. Half of the production waters from each site were filtered immediately after collection and the total genomic DNA was extracted from the filter and stored on dry ice in containers for transport back to the laboratory. They were stored at −20 °C for analysis of the nitrate-reducing community. The other half of the collected samples were transported back to the laboratory in coolers and stored at 4 °C prior to further experiments (Table 1).

2.2. Culture media and procedures

The Hungate technique as described by Bryant (1972) was used in this part of the experiment. Five milliliters of the production water were inoculated into each 120-ml serum bottle containing 80 ml basal culture medium under a stream of pure N_2 gas. All serum bottles were sealed with butyl rubber stoppers and aluminum seals and incubated at 37 °C in the dark for 100 days to evaluate the influences of different organic carbon sources on the diversity of nitrate-reducing microorganisms. The basal culture medium contained (g l^{-1}): NaCl, 1.0; KCl, 1.3; $MgCl_2 \cdot 6H_2O$, 1.0; $CaCl_2$, 0.025; KH_2PO_4, 0.75; $K_2HPO_4 \cdot 3H_2O$, 1.16; NH_4Cl, 0.5; KNO_3, 1.0. Two different groups of organic carbons were used; the first contained mixed organic acids of aspartate (1.0 g l^{-1}) and citrate (8.5 g l^{-1}), while the second contained mixed alkanes (4.0 ml l^{-1}) of C_{15}–C_{20} hydrocarbons.

2.3. DNA extraction

Production water (2.5 l) from these oil reservoirs was filtered on site and microbial biomass was collected for total genomic DNA extraction. Microbial biomass of 20 ml amended culture from incubation was collected by centrifugation at 13,000 x *g* for 1 min. Total genomic DNA of both sets of samples was extracted with the AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Biosciences, California, U.S.) following the manufacturer's instructions.

2.4. PCR amplification of napA genes

Primers for the detection of *napA* genes were based on those previously described (Alcantara-Hernandez, 2009) and synthesized (GenScript, New Jersey). However, the PCR mixture, and the protocol of PCR amplification of *napA* genes were modified according to the production water samples from oil reservoirs. Primer sequences used to amplify *napA* genes were: napAf1: 5′-C TGG ACI ATG GGY TTI AAC CA-3′; napAr1: 5′–CC TTC YTT YTC IAC CCA CAT-3′. The PCR fragments' expected size was 492 bp.

Each 25-μl PCR mixture for amplification of total genomic DNA obtained from both production water and amended cultures contained 12.5 μM of each primer, 50 ng DNA, 10 μl 2x PCR master mix (Lifefeng Biotechnology, Shanghai, China), which was pre-mixed with Mg^{2+}, deoxynucleotide triphosphate, Taq polymerase, and PCR buffer.

The PCR amplification protocol for the *napA* gene consisted of an initial denaturation at 95 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, at 52 °C for 1 min, and at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. Thermal cycling was carried out with a thermal cycler (Bio-Rad Laboratories, California). PCR products were detected by agarose (1.0%) gel electrophoresis and UV transillumination after ethidium bromide staining.

2.5. Cloning and sequencing

Total genomic DNA of samples was used as template in the PCR assay. One hundred nanograms of each *napA* gene fragment was inserted into 1 μl pMD 19-T Simple Vector (TaKaRa Biotechnology, Dalian, China), and transferred into 100 μl competent cell DH5α (Tiangen Biotech, Beijing, China) to establish a *napA* gene library. The universal primers M13 for PCR amplification (GenScript, New Jersey) were: M13f: 5′-TGT AAA ACG ACG GCC AGT-3′; M13r: 5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′. Positive clones of *napA* library were sequenced (GenScript, New Jersey).

2.6. Phylogenetic analysis

VecScreen software was used to remove the sequence segments of vector origin. The OTUs of the sequences were calculated by FastGroupII; the cutoff similarity value used here was 97%. Types of sequences were detected by ORF finder to find all open reading frames. The deduced protein sequences were compared to those available at NCBI Protein Blast.

2.7. Nucleotide sequence accession numbers

The partial sequences (~500 bp) obtained in this study were deposited in the GenBank database under the Accession Numbers HQ727696–HQ727722.

Table 1
Clones obtained and sequenced from different sources.

	Production waters			Organic acids enrichments			Alkane enrichments		
Oil reservoirs	Menggu lin	Zhan 3	Ba 19	Menggu lin	Zhan 3	Ba 19	Menggu lin	Zhan 3	Ba 19
Clones	81	87	90	136	91	135	94	88	92
Total amount	258			362			274		