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New molecular method to detect denitrifying anaerobic methane oxidation bacteria from different environmental niches

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ABSTRACT

The denitrifying anaerobic methane oxidation is an ecologically important process for reducing the potential methane emission into the atmosphere. The responsible bacterium for this process was *Candidatus* Methylomirabilis oxyfera belonging to the bacterial phylum of NC10. In this study, a new pair of primers targeting all the five groups of NC10 bacteria was designed to amplify NC10 bacteria from different environmental niches. The results showed that the group A was the dominant NC10 phylum bacteria from the sludges and food waste digestate while in paddy soil samples, group A and group B had nearly the same proportion. Our results also indicated that NC10 bacteria could exist in a high pH environment (pH 9.24) from the food waste treatment facility. The Pearson relationship analysis showed that the pH had a significant positive relationship with the NC10 bacterial diversity ($p < 0.05$). The redundancy analysis further revealed that the pH, volatile solid and nitrite nitrogen were the most important factors in shaping the NC10 bacterial structure ($p = 0.01$) based on the variation inflation factors selection and Monte Carlo test (999 times). Results of this study extended the existing molecular tools for studying the NC10 bacterial community structures and provided new information on the ecological distributions of NC10 bacteria.

Introduction

Methane is an important greenhouse gas that is, on a molar basis, 28 times more potent than the carbon dioxide (IPCC, 2013). The methane concentration in the atmosphere has increased tremendous from 830 ppb in 1980 to 1799 ppb in 2010 (Kirschke et al., 2013) and still keep increasing 1% annually, thus contributing up to 20% global greenhouse effects.

Microbial mediated methane oxidation is an effective way to prevent the methane emission into the atmosphere. Depending on the existence of oxygen, methane oxidation process can be divided into two major pathways: aerobic oxidation and anaerobic oxidation. The aerobic oxidation uses oxygen as the

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electron acceptor while anaerobic methane oxidation uses other electron acceptors, such as sulfate, nitrate, nitrite and metal oxide. The aerobic methane oxidation has been studied extensively in recent decades such as in paddy soil (Wu et al., 2009), forestry soil (Kolb et al., 2005), sediments (Costello et al., 2002; Rahalkar and Schink, 2007), as well as landfill cover layers (Cebron et al., 2007; Li et al., 2014). The anaerobic methane oxidation, however, is a newly discovered microbial process. The denitrifying anaerobic methane oxidation (DAMO) is one of the anaerobic methane oxidation processes mediated by two major microorganisms, *Candidatus* Methylomirabilis oxyfera belonging to the bacterial phylum of NC10 (Ettwig et al., 2010; Raghoebarsing et al., 2006) and *Candidatus* Methanoperedens nitroreducens belonging to the archaeon order of *Methanosarcinales* (Haroon et al., 2013). Thus far, DAMO archaea had very limited studies due to the difficulties in enrichment and detection (Ding et al., 2015) and most of the studies focused on the NC10 bacteria. The NC10 bacteria have been found in diverse environments, such as in paddy soil (Ding et al., 2016; Shen et al., 2014, 2016; Wang et al., 2012), wetlands (Chen et al., 2015; Hu et al., 2014a; Shen et al., 2015a, 2015b; Zhu et al., 2015), lake sediments (Wang et al., 2016), and wastewater sludges (Luesken et al., 2011). The NC10 bacteria mainly consist of five groups, namely group A, group B, group C, group D and group E (Chen et al., 2014; Ettwig et al., 2009). The group A has been widely detected both in environmental samples and in enriched cultures. In several enrichments, the group A is the dominate group, indicating that it is the "true DAMO" (Ettwig et al., 2009). The group B is the major group in agriculture soils (Shen et al., 2016), but thus far, no enrichment dominating by group B is obtained. There is few information on the group C and group D (Kojima et al., 2012) mainly due to difficulties in detection. The group E is newly recovered from the marine system (Chen et al., 2014, 2015) and thus far, it has not been detected in other environmental niches.

The NC10 specific primers which are currently widely used are developed based on the enrichment culture. The maximum nitrite removal rate was 33.5 mmol/day and group A is the dominant group in this culture (Ettwig et al., 2009). Therefore, these primers are not quite suitable for detecting other groups of NC10 bacteria, especially the group C, group D and group E. Therefore, more sensitive primers are needed to study the NC10 bacteria distribution and ecological significance comprehensively.

The purpose of this study is to develop suitable molecular primers covering all the five group members of NC10 bacteria. The study intends to extend the existing molecular tools for studying the community structures and provides new information on the distributions of NC10 bacteria from different ecological systems.

1. Materials and methods

1.1. Site description and sampling

The wastewater sludges in this study were collected from wastewater treatment plants located at Changchun, Beijing and Kunming City, representing the northern, middle and southern part of China, respectively. The paddy soil sample was obtained in a rice field in suburb of Changping District, Beijing. The sample was collected at approximately 50–80 cm below the surface layer using the stainless steel ring sampler (diameter, 50 mm). Another sample was food waste digestate collected from an anaerobic digestion facility in Beijing. Detailed sample information was listed in Table 1.

Approximately 0.5 kg of each sample was stored in zipper style polyethylene bags on ice and transported to the laboratory in 6 hr for further analysis. The samples were subsequently separated into two parts. The first part was stored at 4°C for the physiochemical analysis within 24 hr and the other part was frozen at −20°C for the following deoxyribonucleic acid (DNA) extraction and microbial analysis.

1.2. Physiochemical analysis

Moisture content and volatile solid (VS) was measured by weighing the residues dried in porcelain crucibles at 105°C for 6 hr and 650°C for 3 hr, respectively. The pH was measured at a liquid-to-solid ratio of 10:1 (*V*/*m*) by pH meter (FE20, Mettler Toledo, Switzerland). Ammonia nitrogen (NH_4^+–N), nitrate nitrogen (NO_3^-–N) and nitrite nitrogen (NO_2^-–N) were extracted by 2 mol/L KCl and determined colorimetrically (DR 6000, Hach, USA). The physiochemical characteristics of the samples were listed in Table S1.

1.3. Primer design

The design of the new primers was based on the 16S ribosomal ribonucleic acid (rRNA) gene alignment of group A to group E of NC10 bacteria and *Candidatus* Methylomirabilis oxyfera. The 16S rRNA gene sequences of these bacteria were downloaded from National Centre for Biotechnology (NCBI) database (accession numbers: NR_102979, JF803481, FJ621558, AB661499, AB486889, DQ906859, AB179508, AF317743, KF742460, KF742470, KM888236). The alignment of these sequences was performed by the Clustal X (version 2.0) software (Larkin et al., 2007) and the conversed region was selected for primer design (Fig. 1). The primer design was then carried out using the Primer Premier software (version 6.0, Premier Biosoft International, USA). Three mismatched bases were found in the alignment and they were changed to degenerate bases in the primers. The forward and reverse primers were designated as XS-F and XS-R (Table 2) and the expected length was 286 bp with an optimal annealing temperature of 56.6°C. The hairpin structure was not existed in this pair of primers and the cross dimer dG was 1.7 kcal/mol. The

Table 1 – Sample information in this study.

Sample	Source	Location	Sampling time
PS	Paddy soil	Beijing	18th July, 2016
FW	Food waste digestate	Beijing	23rd October, 2015
SC	Domestic wastewater sludge	Changchun	27th May, 2016
SK	Domestic wastewater sludge	Kunming	4th August, 2015
SB	Domestic wastewater sludge	Beijing	10th August, 2016

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