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ORIGINAL PAPER

NC10 bacteria promoted methane oxidation coupled to chlorate reduction

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Abstract The strictly anaerobic serum bottles were applied to investigate methane oxidation coupled to chlorate (ClO_3^-) reduction (MO-CR) without exogenous oxygen. 0.35 mM ClO_3^- was consumed within 20 days at the reduction rate of 17.50 μM/d, over three times than that of ClO_4^-. Chlorite (ClO_2^-) was not detected throughout the experiment and the mass recovery of Cl^- was over 89%. Isotope tracing results showed most of $^{13}CH_4$ was oxided to CO_2, and the electrons recovery reached to 77.6%. Small amounts of $^{13}CH_4$ was consumed for DOC production probably through aerobic methane oxidation process, with oxygen generated from disproportionation reaction. In *pMMO* (key enzyme in aerobic oxidation of methane) inhibition tests, ClO_3^- reduction rate was slowed to 7. 0 μmol/d by 2 mM C_2H_2, real-time quantitative PCR also showed the transcript abundance of *pMMO* and *Cld* were significantly dropped at the later period of experiment, indicating that the O_2 disproportionated from ClO_2^- was utilized to active CH_4. NC10 bacteria *Candidatus Methylomirabilis*, related closely to oxygenic denitrifiers *M. oxyfera*, was detected in the system, and got enriched along with chlorate reduction. Several pieces of evidence supported that NC10 bacteria promoted CH_4 oxidation coupled to ClO_3^- reduction, these oxygenic denitrifiers may perform ClO_2^- disproportionation to produce O_2, and then oxidized methane intracellularly.

Keywords Chlorate reduction · Methane oxidation · NC10 · Chlorite disproportionation

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Introduction

Denitrifying anaerobic methane oxidation (DAMO) is an important biochemical process in global nutrient cycles of carbon and nitrogen (Hinrichs et al. 1999; Raghoebarsing et al. 2006; Zhou et al. 2017). It is also regarded as an economical and environmental friendly wastewater treatment process, since microorganisms can use methane produced by anaerobic digestion to remove nitrogen in situ (Knittel and Boetius 2009).

DAMO process is performed through two different pathways: in "reverse methanogenesis" pathway, anaerobic methanotrophic archaea (ANME) utilizes CH_4 to generate electrons via reverse methanogenesis, and then reduces nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase (*Nar*) (Haroon et al. 2013; Raghoebarsing et al. 2006); in "intra-aerobic" pathway of a specific nitrite-dependent DAMO (n-DAMO) reported by Ettwig et al. (2010), the NC10 bacteria *M. oxyfera* reduce NO_2^- to NO by nitrite reductase (*Nir*), and then disproportion NO to N_2 and O_2 by a proposed NO dismutase, and utilize the generated O_2 to oxidize methane intracellularly by a membrane-bound methane mono-oxygenase (*Pmo*). The disproportionation of NO by *M. oxyfera* was proved by isotope tracing as the fourth way of oxygen production, along with photosynthesis, chlorate respiration, and reactive oxygen species cluster. According to the calculation, 3/4 of the self-produced O_2 was used for the oxidation of CH_4, and the remaining 1/4 O_2 was transferred to the terminal oxidase to realize its own carbon and nitrogen cycle (Wu et al. 2011).

Many other oxyanions were found able to be reduced driven by methane oxidation recently, including chromate, selenate, and perchlorate, researchers proposed similar methane oxidation pathway since oxygen was usually not detectable in their studies (Lai et al. 2016b, 2018c; Lv et al. 2020, 2019; Shi et al. 2019). Among those oxyanions, perchlorate has the most similar bio-reduction pathway as nitrate (Coates and Achenbach 2004). Perchlorate reducing bacteria (PRB) reduce ClO_4^- and chlorate (ClO_3^-) to chlorite (ClO_2^-) by perchlorate reductase (*Pcr*), a molybdopterin-containing respiratory reductase resembling *Nar* (Ontiveros-Valencia et al. 2014; Chen et al. 2016), ClO_2^- is later disproportionated to Cl^- and O_2 by chlorite dismutase (*Cld*) (Bardiya and Bae 2011). Though researchers found methane oxidation coupled to perchlorate reduction (MO-PR) may through reverse methanogesis pathway (Luo et al. 2015; Lv et al. 2019; Xie et al. 2018), it is actually not clear whether the O_2 produced by ClO_2^- disproportionation also makes difference in methane activation process or not.

Since *Cld* has the potential of creating an "intra-aerobic" environment, which is essential for certain microbial process. However, the distribution of *Cld* shows little correlation with its function. Most of denitrifiers could reduce ClO_4^- or ClO_3^- to ClO_2^-, but not able to reduce ClO_2^- to Cl^- because of chlorite dismutase absence (Lindqvist et al. 2012). In other situations, chlorite dismutase were found in bacteria and archaea that cannot grow with chlorate as electron acceptor, e.g., in the nitrite-oxidizing genera *Nitrospira* (Maixner et al. 2008) and *Nitrobacter* (Mlynek et al. 2011). The role of *Cld* in these organisms, however, is unclear.

To investigate if the NC 10 bacteria can link the methane oxidation coupled to chlorate reduction process and function as chlorite dismutase, we carried out $^{13}CH_4$ isotope tracing and functional enzymes inhibition experiments to verify the MO-CR pathway. Real-time qPCR (RT-qPCR) and phylogenetic analysis were applied to evaluate the transcript abundance of key functional genes and define the status of the dominant bacteria, respectively. These results may broaden our understanding of the oxygenic denitrifiers and provide a microbial perspective for (per)chlorate removal.

Materials and methods

Experimental setup and operation

We set up two CH_4-based membrane biofilm batch reactors (CH_4-MBBR) described in Lv et al. (2019) for enrichment cultivation, and fed them with perchlorate and chlorate, respectively. The temperature was kept stable (35 ± 1 °C) for all experiments. When the reduction rate of substrate became stable, approximately 20 mL of the enriched culture were inoculated into 120-mL serum bottles with 50 mL of anoxic medium, specific parameter settings were shown in Table S1. Bottles in triplicate were first sparged with argon, then closed with butyl rubber stoppers, and finally sealed with crimped aluminum caps. $^{13}CH_4$ and ClO_3^- were introduced as the sole electron donor and acceptor, respectively. After ClO_3^- was completely consumed, it was introduced again along with two enzyme inhibitors, 2 mM 2-bromoethanesulphonate (BES), known as a potent inhibitor of methanogenesis (Waghmode et al. 2015), thus can rule out the influence of reverse methanogesis pathway, and 2 mM acetylene (C_2H_2), known for effective inhibit of the key functional enzyme *Pmo* of aerobic methane oxidation (Kits et al. 2015), respectively, to explore the contribution of methanotrophic archaea and

bacteria to CH_4-dependent ClO_3^- reduction. At the beginning (day 0), middle (day 25) and end of the test (day 40) microbial consortia were sampled for molecular analysis.

Chemical analyses

For each serum bottles, liquid samples were taken using 1 mL syringes and were filtered immediately through a 0.22-μm membrane filter (LC + PVDF membrane, Shanghai Xinya, China) to eliminate the influence of suspended microorganism. ClO_3^-, ClO_2^-, and Cl^- was measured using ion chromatography (DIONEX ICS-1000, USA) with an AS 19 column, the eluent concentration of 20 mM KOH and a 1 mL/min flow rate. DOC and DIC was detected by GC-IRMS isotope mass spectrograph (Thermo Scientific, USA) by Third Institute of Oceanography, MNR (Xiamen, China).

O_2 in the headspace of the serum bottles was measured with gas chromatography (Agilent Technologies GC system, model 7890A, Agilent Technologies Inc., USA), and the gas-phase concentration was used to calculate the dissolved O_2 concentration in liquid phase according to Henry's Law (Campbell and Brand 1998). $^{13}CH_4$ and $^{13}CO_2$ was measured with GC–MS (GC system, model 7890A; MS system, model 5977B). pH values, which were monitored by a portable pH meter (Seven Easy, Mettler Toledo, Switzerland) throughout the experiment, were between 7.0 and 7.7 for all stages.

Gene clone library and illumina sequencing

Primers 1051F (5′-ARCGTGGAGACAGGTGGT-3′) and qP2R (5′-CTCAGCGACTTCGAGTACAG-3′) were used to amplify the 16S rRNA gene for NC10 bacteria. PCR amplification was performed using the following program: the temperature was first maintained at 94 °C for 3 min, after that, a sequence of stages including denaturation (94 °C for 30 s), annealing (62 °C for 40 s) and extension (72 °C for 40 s) were repeated for 35 cycles, and subsequently, a final extension stage was performed at 72 °C for 10 min. The PCR products were then purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The purified amplicons were cloned in competent *E. coli* Trans-T1 by using the *pEASY*-T1 cloning vector (TransGen Biotech, Beijing, China). 50 clones, which were randomly picked, were tranferred from solid LB medium with 100 μg/mL ampicillin to liquid LB medium, and were grown overnight for afterwards sequencing. Phylogenetic analysis was based on aligned homologous nucleotides as reported by Suau et al. (1999). ClustalX (Thompson et al. 1997) was chosen to align the sequences and guided tree files were utilized to create phylogenetic trees. The constructed trees were basing on the neighbor-joining method implemented in MEGA 6.0 (Tamura et al. 2007).

Primers 341F (5′-CCTAYGGGRBGCASCAG-3′ and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were chosen for amplification of a 466-bp fragment contained in the bacterial 16S rRNA gene flanking the V3 and V4 regions. The purified amplicons were send to Novogene company (Beijing, China) to process Illumina MiSeq sequencing with standard protocols and the data was processed using QIIME (version 1.7.0) pipeline (Caporaso et al. 2010).

Quantification of key functional genes

The plasmids containing target fragments were constructed and used to generate standard curves based on serial dilutions containing series of target gene copies. Primers that target the perchlorate reductase gene (*pcrA*), as well as the 16S rRNA gene for archaea, and methyl coenzyme M reductase gene (*mcrA*) were used for plasmid construction The names of primer, as well as their sequences, and the PCR conditions for each target gene mentioned above are shown in Table S2. We used SYBR Premix Ex Taq Kit (Takara Bio Inc, Japan) and performed qPCR as previously described by Zhao et al. (2011). Water instead of template DNA in the PCR reaction mixture was performed as negative controls. Triplicate PCR reactions were performed for all samples and negative controls. Along with the slopes of the plasmid standard curves, efficiency values for quantification by qPCR are also shown in Table S2, too.

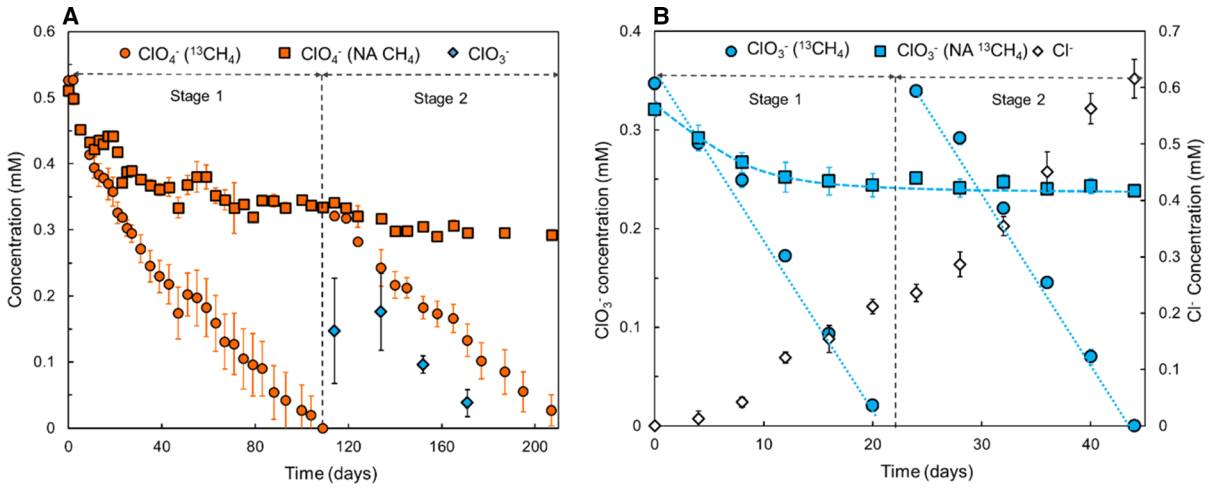

Fig. 1 Perchlorate (**a**) and chlorate (**b**) reduction in $^{13}CH_4$ supplied serum bottles

Results and discussion

The kinetics of perchlorate and chlorate reduction

Figure 1a, b show the ClO_4^- and ClO_3^- reductions in serum bottles fed with $^{13}CH_4$, respectively. In both experiments, $^{13}CH_4$ were not the rate-limiting substrate, since the actual $^{13}CH_4$ concentration were far higher than the maximum theoretical $^{13}CH_4$ demand throughout experiment according to Eqs. (1) and (2) (Table S3). 0.53 mM ClO_4^- was completely reduced in 109 days in stage 1, the reduction rate of ClO_4^- was 4.82 μM/d, which was far lower than the reduction rate of perchlorate in MBBR (18.06 μM/d) reported by Lv et al. (2019) in similar circumstance. This might due to lower methane utilization efficiency and less biomass in serum bottles (Lu et al. 2016; Shi et al. 2020b) compare to MBBR with higher methane delivery and utilization efficiency. 0.32 mM ClO_4^- was added in stage 2 and reduced completely within 93 days. ClO_3^- accumulation was detected at the beginning of stage 2 and was consumed following ClO_4^- reduction as Lv et al. (2019) reported. In the control groups without methane supply, about 0.20 mM ClO_4^- was removed at much lower rate of 2.86 μM/d in the first 70 days and remained steady in the rest of time. The combine result suggested that methane indeed served as electron donor for perchlorate reduction, small amount of ClO_4^- reduction in control groups may be driven by carbon source produced from microbial cell degradation (Lai et al. 2018a).

$$ClO_4^- + CH_4 \rightarrow HCO_3^- + H^+ + Cl^- + H_2O \quad (1)$$

$$1.33ClO_3^- + CH_4 \rightarrow HCO_3^- + H^+ + 1.33Cl^- + H_2O \quad (2)$$

As shown in Fig. 1b, compared with ClO_4^-, the reduction rate of ClO_3^- was much faster. 0.35 mM ClO_3^- was consumed within 20 days at the reduction rate of 17.5 μM/d, three times higher than that of ClO_4^-. This is probably because that the reduction of ClO_4^- to ClO_3^- was the speed limiting step (Dudley et al. 2008; Zhang et al. 2016), and the reduction of ClO_3^- required less electrons. After ClO_3^- was removed completely in stage 1, another 0.35 mM ClO_3^- was supplied in stage 2 and completely reduced at the same rate of stage 1. ClO_2^- was not detected throughout the experiment, while the final reduction product, Cl^-, constantly increased in parallel to ClO_3^- reduction. Cl^- reached 0.62 mM at the end of experiment, which was 89.70% of the theoretical Cl^- yield from complete reductive dechlorination of all input ClO_3^-. Since denitrifying bacteria cannot disproportionate ClO_2^- independently, the complete reduction of ClO_3^- may involve other microorganisms (Bardiya and Bae 2011).

Electronic equilibrium in $^{13}CH_4$-dependent chlorate reduction

$\delta^{13}C$ value of dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) were measured to calculate the electrons balance during the methane oxidation coupled to perchlorate and chlorate reduction processes. As showed in Fig. 2, the total amount of ClO_3^- reduction was about 55 µmol, which required 41.25 µmol $^{13}CH_4$ theoretically if all the methane was reduced to CO_2, according to Eq. (2). Besides, according to Fig. 2, $\delta^{13}C$ value of DIC rises proportionally at an average ClO_3^-/DIC ratio of about 1.72 as the amount of ClO_3^- declines, close to the theoretical number of 1.33, calculated according to Eq. (2). The electrons recovery rate was about 77.6%, lower than reported by Ettwig et al. (2010) in nitrate-dependent anaerobic methane oxidation. However, $\delta^{13}C$ value of DOC was also detected to be increasing along with ClO_3^- reduction and accumulated up to ~ 45 µmol at day 20, while ^{13}DOC was rarely detected in $^{13}CH_4$ -dependent ClO_4^- reduction (Fig. S1). The generation of DOC compensated the slight difference between theoretical number mentioned above. More DOC was produced might because of oxygen concentration increased from the disproportionation of ClO_2^-, Wei et al. has reported that microorganisms are prone to produce more DOC when oxygen concentration is slightly enhanced in anoxic condition (Wei et al. 2016).

Kinetics and functional genes transcript abundance of ClO_3^- reduction in inhibition tests

To explore whether aerobic methane oxidation process was involved in MO-CR, BES and C_2H_2 were applied to inhibit Mcr and Pmo in inhibition tests. Figure 3a showed that compare to the control group without inhibitors, ClO_3^- reduction was significantly inhibited in the presence of 2 mM BES, indicating that archaea was possibly involved in the MO-CR. It is consistent with the studies reported by Lu et al. (2016) and Shi et al. (2020b). Interestingly, ClO_3^- reduction rate was slowed to 7.0 µmol/d when C_2H_2 was presented. C_2H_2 is known for effective inhibit of the key functional enzyme *Pmo* of aerobic methane oxidation (Kits et al. 2015). Since serum bottles were in strictly anaerobic condition and no oxygen intrusion was detected throughout the whole experiment. The result indicates that oxygen was probably produced in the serum bottle during the MO-PR by certain microorganisms, and NC10 bacteria, proved to be producing oxygen in DAMO process (Ettwig et al. (2012)), is highly probable of playing the same role in MO-PR process.

The result was supported by the changes of transcript abundances of *pcrA*, *Cld*, and *pMMO* too.

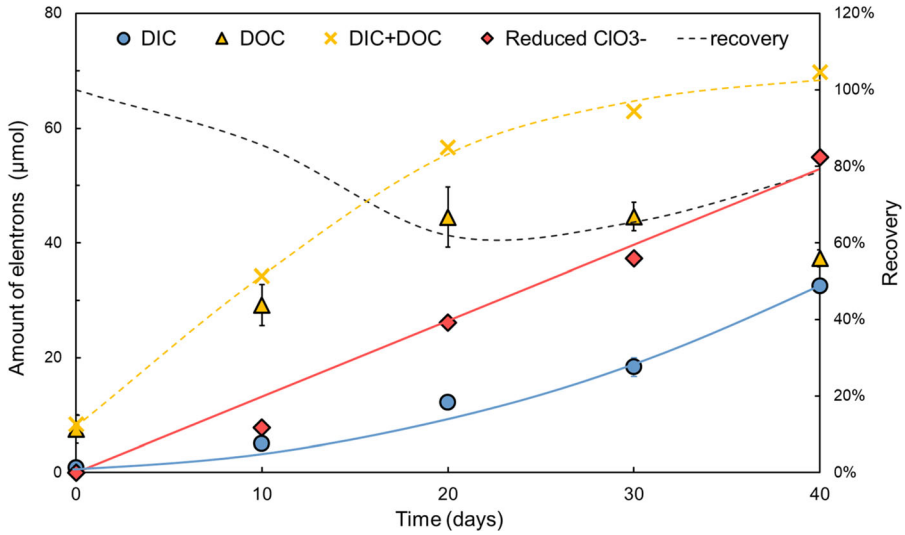

Fig. 2 Recovery and amounts of electrons in methane oxidation and chlorate reduction processes. DIC, DOC are abbreviations of dissolved inorganic carbon and dissolved organic carbon, respectively

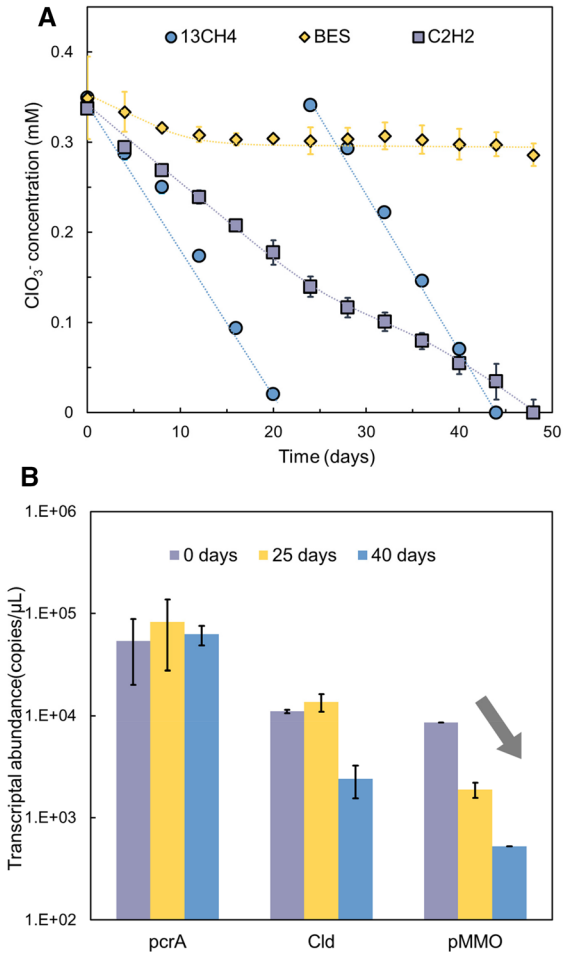

Fig. 3 Inhibition tests: **a** chlorate reduction in groups of $^{13}CH_4$, inhibitors BES and C_2H_2; **b** transcript abundances of functional genes in C_2H_2 groups. The grey arrow represents the declining trend of transcript abundance over time

As shown in Fig. 3b, the *pcrA* transcript abundances kept stable when 2 mM C_2H_2 was in presence, while the transcript abundance of *pMMO* dropped significantly from 8.60×10^3 to 5.24×10^2 copies/μL at the end of experiment. Similarly, *Cld* transcript abundance decreased from 1.36×10^4 (day 25) to 2.40×10^3 copies/μL (day 40) as well. Combined with the above chlorate reduction kinetics, we propose that there must be certain microbes utilizing O_2 disproportionated from ClO_2^- to active CH_4 in anaerobic serum bottles. Ettwig et al. (2012) found oxygen producing bacteria, *M. oxyfera*, mediated the process of n-DAMO. However, it is not clear yet which oxygen producing bacteria play the role in MOPR.

Evolution of microbial community

Figure 4 showed the bacterial abundance at the species level of inoculum and the final sample of experiment. As the inoculum was from methane-fed denitrification system, *Ignavibacterium album* accounted about 6.0% in the beginning and decreased to ~ 3.2% at the end of experiment. The *Ignavibacterium album is* a heterotrophic bacteria able to utilize organic compound as carbon and electron donor, it's abundance decreased might because the residual organics were all consumed and no organics were introduced throughout the experiment (Hatamoto et al. 2014). *Chloroflexi bacterium* was reported to be enriched in n-DAMO enrichment culture (Ettwig et al. 2010), but its function remains unknown. Methanotrophs *Methylocystis parvus* were enriched along with chlorate reduction as numerous researchers have shown the methanotrophs are responsible for aerobic activation of CH_4 (Kits et al. 2015; Lai et al. 2018b, 2016a). In this case, methanotrophs may consume CH_4 and O_2 disproportionated from ClO_2^-, according to Lv et al. (2019). Besides, another methanotrophs *Candidatus Methylomirabilis*, which belongs to NC10 phylum, was also detected and got enriched. Most NC10 bacteria are anaerobic methanotrophs, and are present in various natural and engineering systems, such as wetlands, rivers, lakes, rice paddies, tidal zones, and wastewater treatment plants (Padilla et al. 2016; Shen et al. 2016). *M. oxyfera* was known to be able to produce O_2 intracellularly through NO disproportion, thus carry out DAMO process independently (Ettwig et al. (2012)). Besides, Hatamoto et al. (2017) found a novel genus of the NC10 phylum being dominant following enrichment cultivation in a DAMO reactor. Since chlorate was vigorously reduced during the experiment, both methanotrophs, including one belongs to NC10 phylum, which were enriched throughout the process, are likely to couple methane oxidation to chlorate reduction.

Candidatus *Methanoperedens*, known as ANME-2d, accounted for 24.3% of archaea in inoculum and decreased to ~ 16% in final at genus level. Candidatus *Methanoperedens* was also detected in enriched culture of DAMO. Raghoebarsing et al. (2006) firstly reported that DAMO enrichment included two types of functional microorganisms: the dominant group belonged to NC10 phylum (Ettwig et al. 2010), and

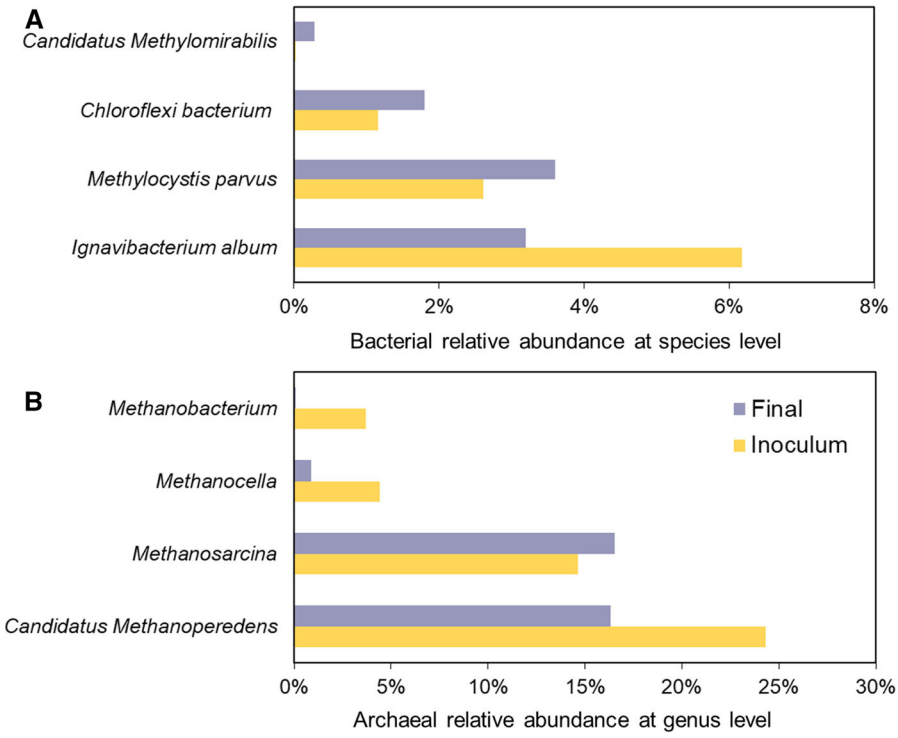

Fig. 4 Relative abundances of bacteria at species level

the other is ANME-2d, known as "*Candidatus Methanoperedens nitroreducens*", which was later confirmed by Haroon et al. (2013). Ettwig et al. (2008) found that when they altered the substrate from NO_3^- to NO_2^-, the abundance of ANME-2d gradually decreased until it could not be detected, which was consistent with our findings. It was speculated that n-DAMO process could be independently undertaken by *M. oxyfera*, while ANME-2d played key roles when NO_3^- served as electron donor (Haroon et al. 2013). In previous studies, researchers found *Methanosarcina*, related to ANME-3, played essential roles in ClO_4^--dependent methane oxidation (Lv et al. 2019; Shi et al. 2020a; Xie et al. 2018). Likely, NC10 bacteria may also play a significant role in ClO_3^--dependent methane oxidation.

To further investigate the oxygen generation mechanism in MO-CR, 16S rRNA gene of NC10

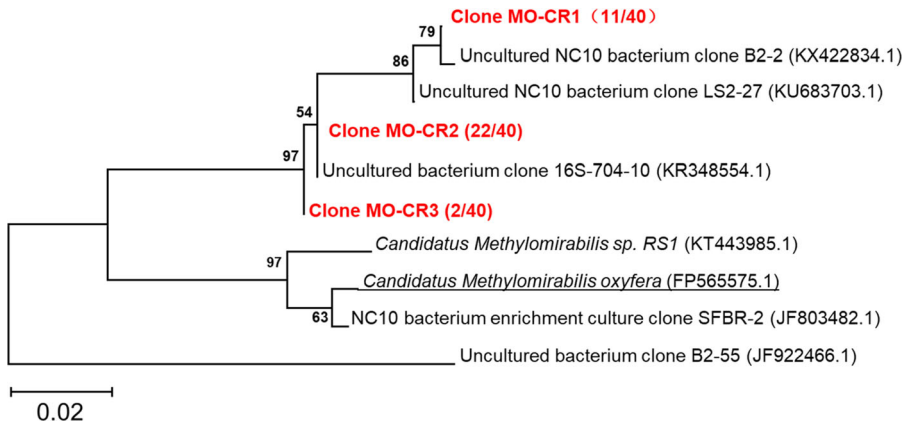

Fig. 5 Phylogenetic relationships among the dominant NC10 bacterial 16S rRNA genes in serum bottles. The evolutionary history was inferred using the neighbor-joining method. The clones are highlighted in red bold

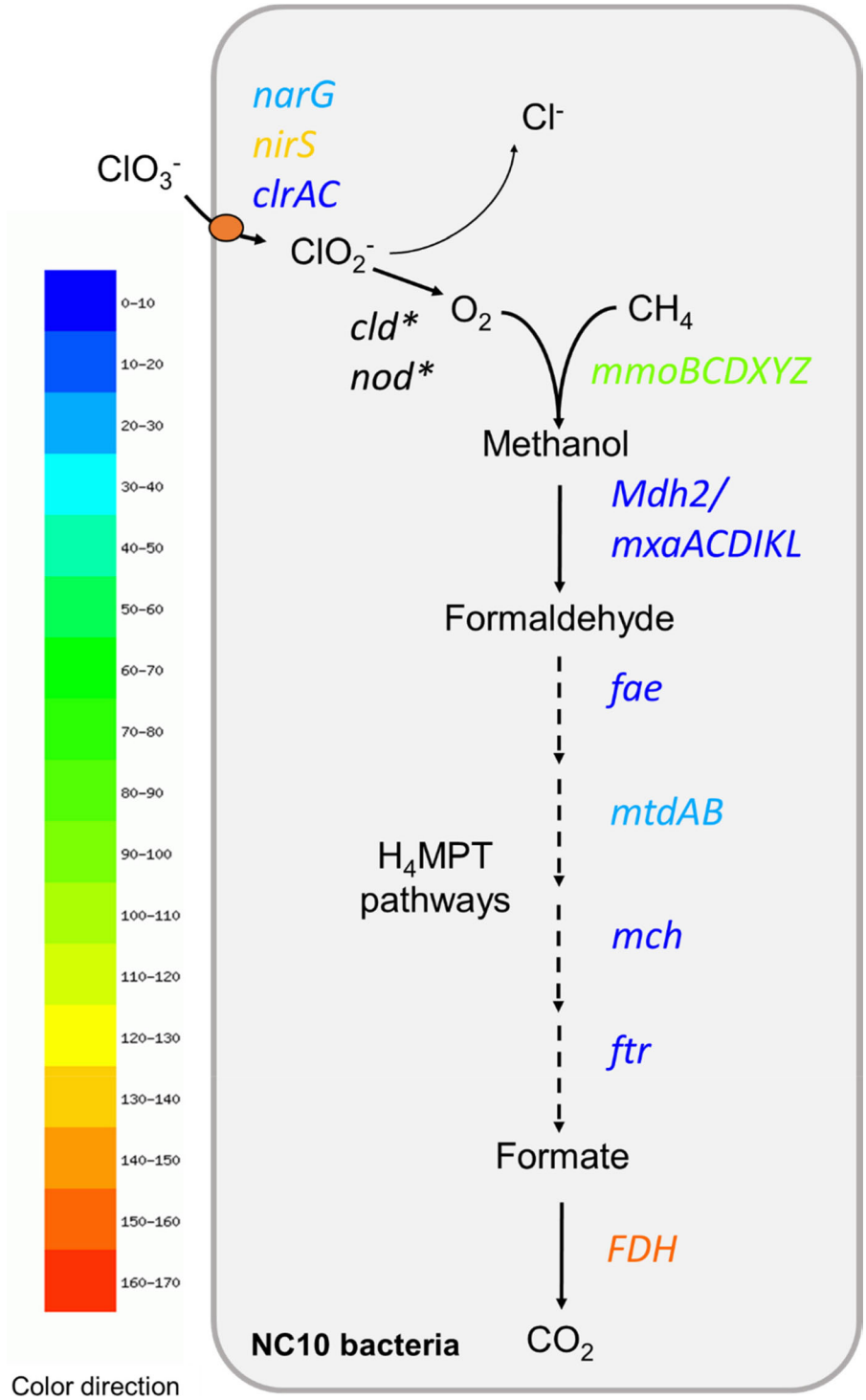

Fig. 6 Proposed pathways of MO-CR based on functional genes prediction. Different colors indicate different numbers of functional genes. * The function genes are not included in Kyoto Encyclopedia of Genes and Genomes (KEGG) database

bacterial clone libraries were constructed. 35 clones were successfully sequenced and analyzed using phylogenetic tree. Figure 5 showed that 11 clones (31.4%) had sequences close to uncultured NC10 bacterium found in wetlands and intertidal zones. Another 24 clones were close to *Candidatus Methylomirabilis oxyfera* (*M. oxyfera*), which could oxide CH_4 using O_2 disproportionated from NO (Ettwig et al. 2010; Zedelius et al. 2011). Recently, NC10 bacteria has been suggested to be involved in the oxidation of methane, alkanes (C6-C30) and benzene (Atashgahi et al. 2018; Ettwig et al. 2010; Zedelius et al. 2011). Considering that NC10 bacteria was enriched in the period of chlorate reduction, we proposed that NC10 bacteria participated in chlorite disproportionation and aerobic methane oxidation using certain enzyme,with NO dismutase or *Cld* being the most probable candidate (Kool et al. 2012).

Proposed pathways of MO-CR mediated by NC10 bacteria

Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, we proposed the pathway of MO-CR mediated by NC10 bacteria, displayed in Fig. 6. Once ClO_3^- is transported into NC10 bacteria, enzymes like *narG*, as well as *nirS*, and *clrAC* can perform a preliminary reduction and produce ClO_2^-. ClO_2^- subsequently disproportionates to O_2^- and Cl^- in the effect of nod or *Cld*, thus creats an "intra-aerobic" environment for aerobic methane oxidation. Methane, consequently, is oxidized to methanol and further to formaldehyde under the action of *mmoBCDXYZ* and *Mdh2*/*mxaACDIKL*, respectively. Formaldehyde is further oxidized in a H_4MPT pathway to form formate, and finally to CO_2 in the effect of *FDH*. All the genes encoding functional enzymes involved in denitrification (DN) process and aerobic methane oxidation (AMO) process were found in our system (except genes encoding *Cld* and *Nod*, which are not included in KEGG). Moreover, genes encoding *nirS*, *MMO*, and *FDH*, were relatively abundant in the system, *nirS* and *MMO* are essential enzymes involved in denitrification and aerobic methane oxidation process (Lawton and Rosenzweig 2016; Zhang et al. 2019), while *FDH* is an essential enzyme catalyzing formate in AMO process (Alrashed et al. 2018).

Conclusions

In this study, we found that microorganisms could reduce chlorate at comparatively high rates using methane as sole electron donor and carbon source. For both ClO_3^- and ClO_4^-, isotope tracing results showed most of $^{13}CH_4$ was oxidized to CO_2, while small part of $^{13}CH_4$ was used for DOC production probably through aerobic methane oxidation process. Functional enzymes inhibition tests confirmed that aerobic methane oxidation occurred along with reverse methanogenesis. NC10 bacteria was enriched along with chlorate reduction, and was regarded as participants that promoted MO-CR. These findings may broaden our understanding of microbial methane oxidation and (per)chlorate reduction, as well as denitrification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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