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Methane-dependent complete denitrification by a single *Methylomirabilis* bacterium

In the format provided by the authors and unedited

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Supplementary Notes

Supplementary Note 1

Reasons for absence of ANME-2d archaea at the enrichment stage

Surprisingly, ANME-2d archaea were almost absent (very low abundance) at the period of enrichment fed with nitrate and methane, which was unusual compared to previous studies[1-3]. Here, we provided some details of culture condition to explain the phenomenon. Membrane Reactor (MBR) and Membrane biofilm Reactor (MBfR) are usually used for cultivation of ANME-2d archaea as for a better anoxic condition with more soluble methane. The partial pressure of methane in MBR and MBfR achieves 100-300 kPa and the soluble methane concentration is always greater than 1 mmol L^{-1} [2-5]. Here, in our GLSBR system, the methane partial pressure is always below 50 kPa and the methane concentration is from 0.1 to 0.7 mmol L^{-1} (Supplementary Fig. 6a). The affinity of the archaeal MCR protein complex requires mM (usually > 1 mmol L^{-1}) concentrations of methane, while the affinity of the bacterial pMMO system is in the μM range[6-10]. Obviously, the methane supply in our SBR-type system is better for cultivation of methanotrophic bacteria rather than archaea. Moreover, approximately 0-8 μmol L^{-1} of oxygen is detected as the reactor inevitably runs with trace amounts of oxygen while medium replenishing (Supplementary Fig. 6b). The oxygen has apparently no negative effect on *Methylomirabilis* bacteria but inhibit ANME-2d archaea by deactivating

MCR which do not tolerate oxygen. This is another key reason explaining the absence of ANME-2d archaea in our enrichment system as a similar situation exited in previous work[11].

Supplementary Note 2

Description of '*Candidatus* Methylomirabilis sinica'

Etymology. *Methylomirabilis*. methyl (new Latin): the methyl group; *mirabilis* (Latin): astonishing, strange. *sinica* (Latin): China. The name implies an organism first discovered in China capable of consuming methane and reducing nitrate via nitrite to dinitrogen gas.

Locality. Enriched and purified from a paddy soil in Hangzhou, China.

Diagnosis. Bacterium of the candidate division NC10 that perform methane oxidation coupled to full denitrification. Grows anaerobically but produces oxygen for methane oxidation. Reduces nitrate via nitrite to dinitrogen gas. Gram-negative rod with diameter of 0.2-0.6 μm and a length of 0.7-2.0 μm, tending to form aggregates. Mesophilic with regard to temperature and pH (cultured at 30-35 ℃ and pH 7-8). Adapted to slow growth (doubling time ~21 days) in oligotrophic habitats on the basis of a high affinity for nitrate compared to most of denitrifiers. Owing to the extremely low growth rate/yield, deposition and maintenance of this strain in culture collections seems difficult and qPCR is needed for growth tracking. Therefore, we opt to tentatively use the provisional *Candidatus*

status[12]. In the future, we will try to cultivate enough cell mass and provide sufficient biological data for meeting the current standards for validly proposing a name for this strain.

Supplementary Note 3

How does '*M. sinica*' solve the problem of redox imbalance?

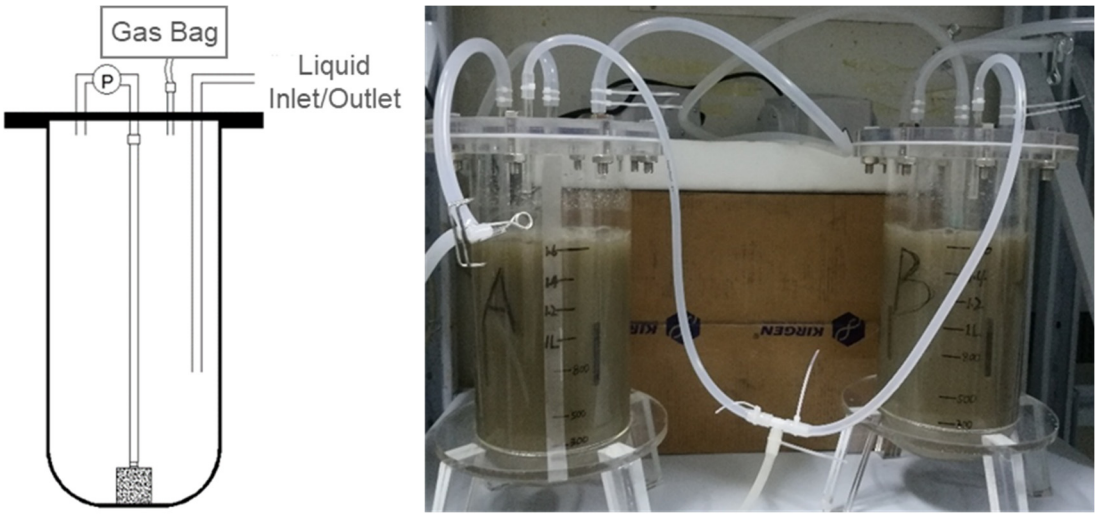
As discussed in the previous study, methane-dependent complete denitrification by '*M. oxyfera*' alone is not feasible for two reasons[13]. One is that the conversion of eight molecules of nitrate only generates four molecules of oxygen, which does not sustain the activation of five methane molecules. Another is that the number of electrons (20) generated from the oxidation of five methane molecules is insufficient for the complete reduction of eight nitrate molecules (24) (Extended Data Fig. 8a)[13]. '*M. sinica*' seems to solve this problem. As trace oxygen was present in enrichment cultures previously, the additional oxygen was considered to participate in this reaction, which activated more methane to balance the electron flows. If so, the formation ratio of CO_2 and N_2 should be close to 5:3 (eq. 1). However, in the enrichment system with trace oxygen, the ratio ($r^{13}CO_2$:$r^{30}N_2$) was estimated as 1.23, which was more close to 5:4 (eq. 2). To give more accurate details whether oxygen is the solution for '*M. sinica*', isotopic labelling tests under strict anoxic conditions were carried out on the highly purified culture (Fig. 3, Extended Data Fig. 4, 5 and

Supplementary Fig. 5). The result showed a ratio ($r^{13}CO_2$:$r^{30}N_2$) of 1.26 ± 0.07 that was more close to 5:4 as well (eq. 2). Therefore, it is strongly indicated that electrons acquired for nitrate reduction to dinitrogen are only from methane and oxygen is probably not necessary. In aerobic methanotrophs, methane is activated to methanol by the insertion of an oxygen atom from O_2 and another oxygen atom is reduced to water, consuming two electrons[14]. It was assumed that electrons for particulate methane monooxygenase (pMMO) were from methanol dehydrogenase (Mdh) (Extended Data Fig. 8a). Here, we propose another possible pathway of electron flows in '*M. sinica*' (Extended Data Fig. 8b). It is likely that only 0.5 molecules of O_2 are required to activate 1 molecule of CH_4 without formation of H_2O, which is thermodynamically exergonic[15]. Then, the electrons from Mdh may flow to periplasmic nitrate reductase (Nap) instead of pMMO, which results in sufficient electron generation (30) from methane oxidation for complete denitrification (24).

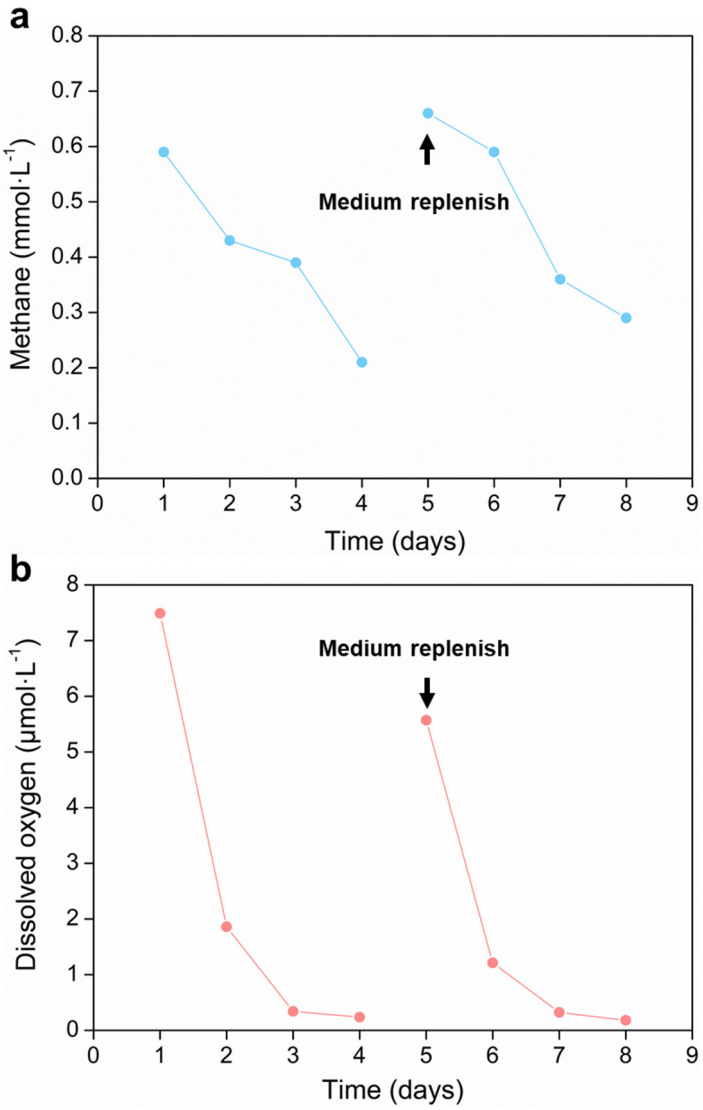
$$10CH_4 + 12NO_3^- + 5O_2 + 12H^+ \rightarrow 10CO_2 + 6N_2 + 26H_2O \quad \text{(eq. 1)}$$

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \quad \text{(eq. 2)}$$

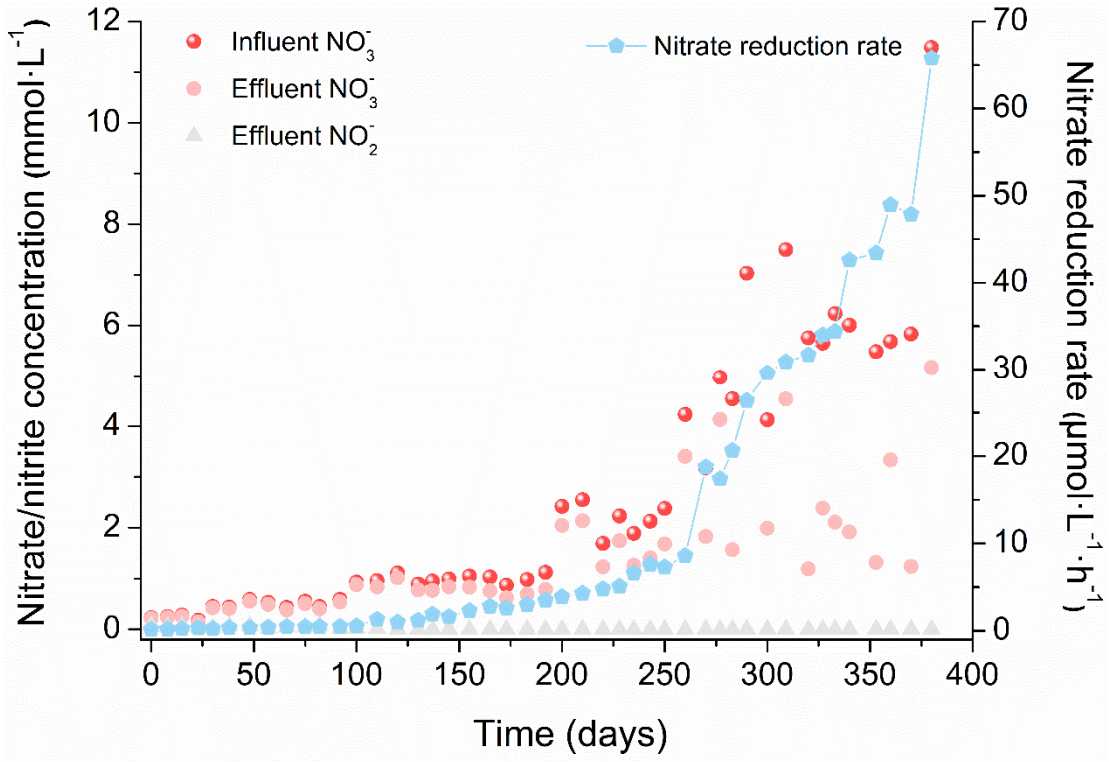
Supplementary Figures



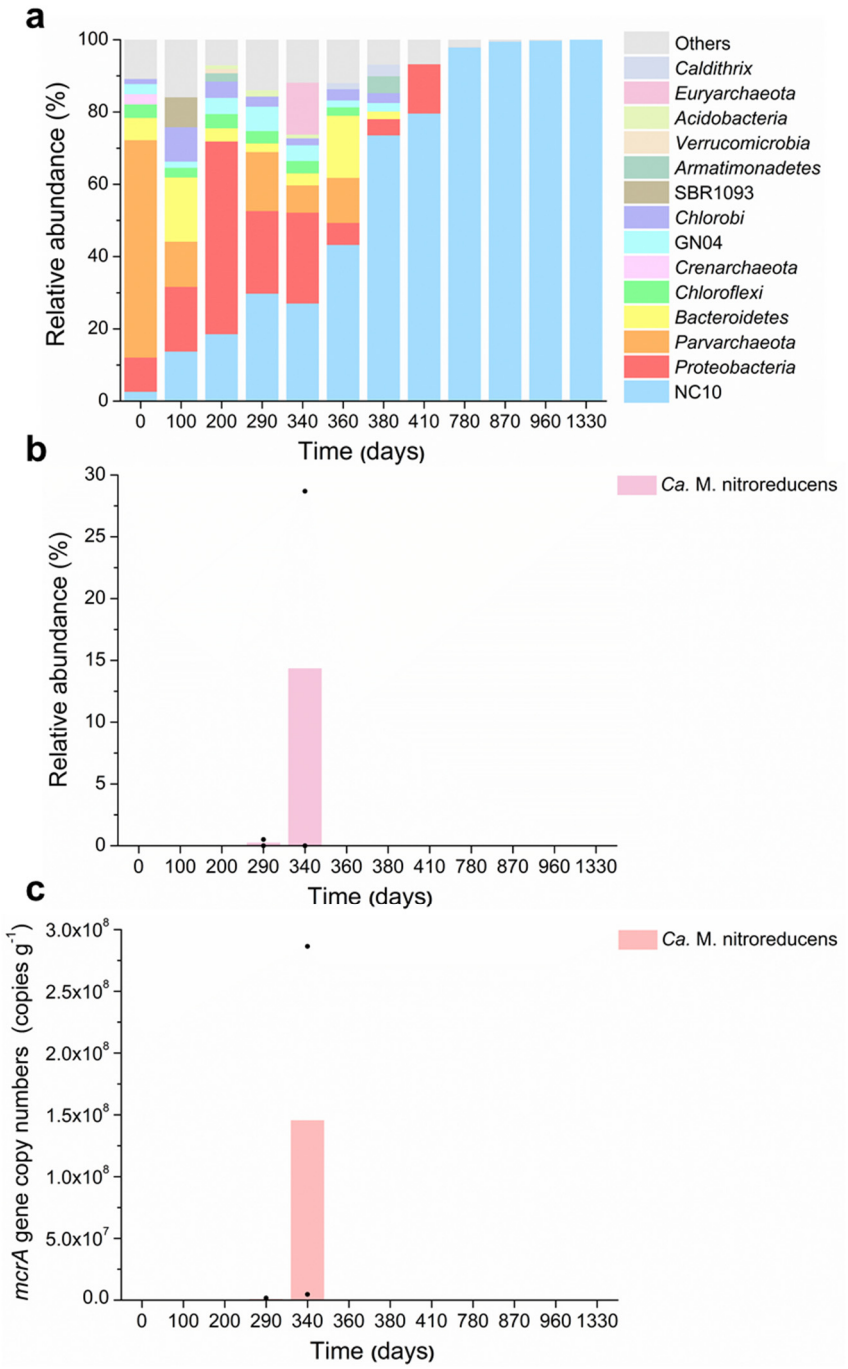
Supplementary Figure 1: Schematic (left) and physical diagrams (right) of GLSBR system.



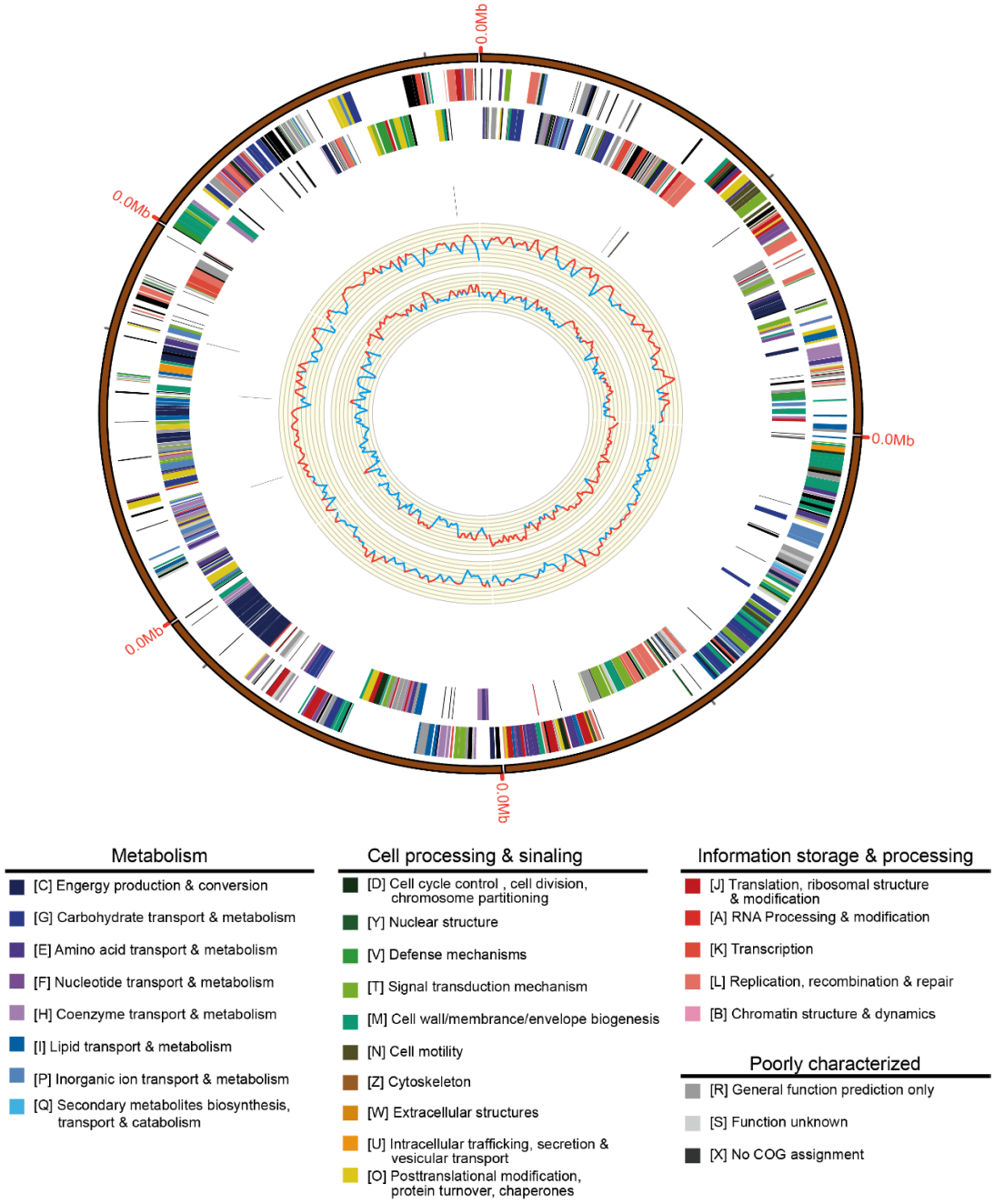
Supplementary Figure 2: Typical dynamics of methane and oxygen levels in the GLSBR at the enrichment stage. Data are presented as mean values (n = 2).



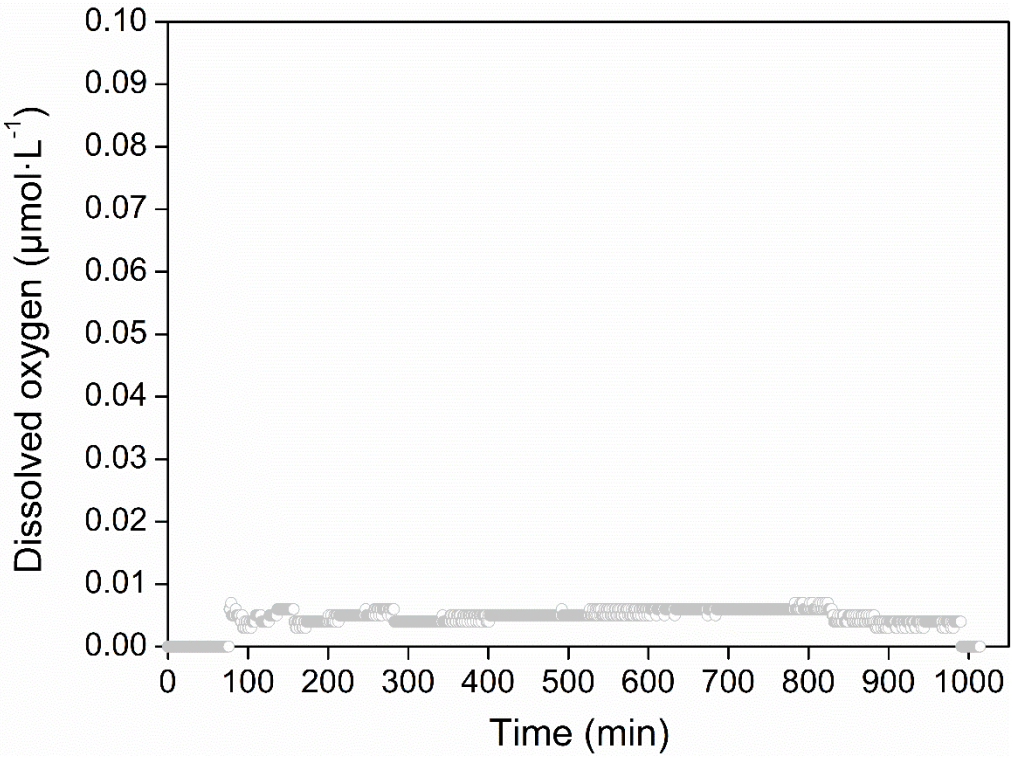
Supplementary Figure 3: Long-term performance data of GLSBR from day 0 to 380. Red circles, pink circles and grey triangles indicated the concentrations of influent nitrate, effluent nitrate and effluent nitrite, respectively. The blue pentagons showed the nitrate removal rates. The nitrite was almost absent throughout the long-term operation. The interval of supernatant replacement was set from two to six days.



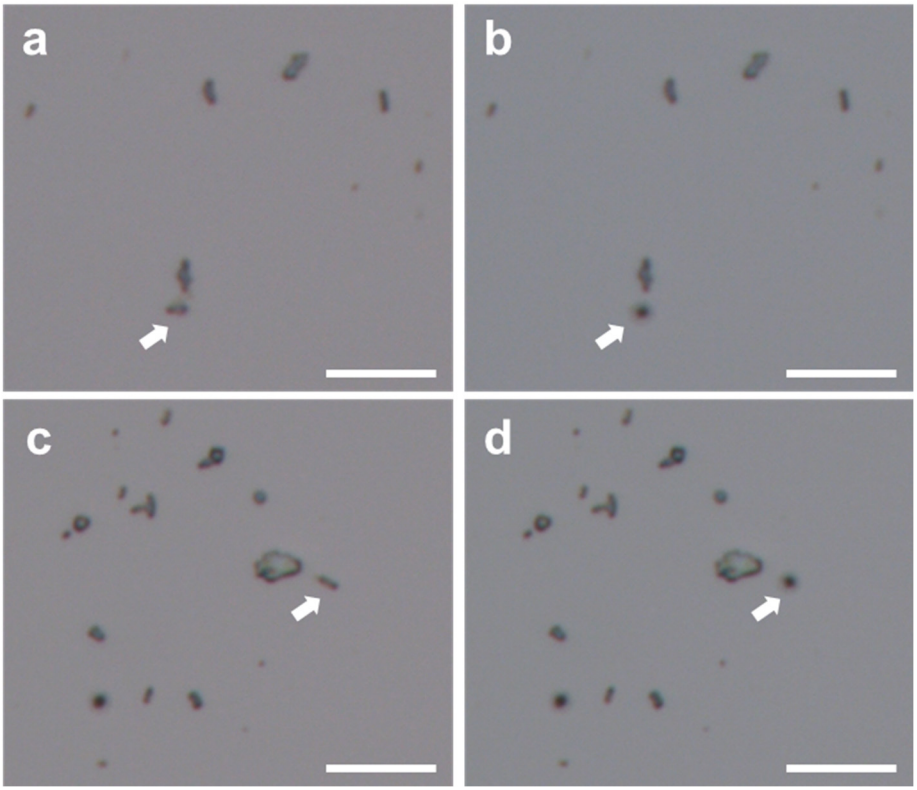
Supplementary Figure 4: Microbial community dynamics of enrichment cultures and purified cultures from day 0 to day 1330. a, Microbial community composition based on the 16S rRNA gene amplicon sequencing. **b,** Relative abundance of methanotrophic archaea '*Ca.* M. nitroreducens' based on the 16S rRNA gene amplicon sequencing. **c,** *mcrA* gene copy numbers of methanotrophic archaea '*Ca.* M. nitroreducens'. Data are presented as mean ($n = 2$) from day 0 to day 380 and individual data points are shown by black circles.



Supplementary Figure 5: Circular representation of '*Ca.* Methylomirabilis *sinica*' genome. This is a consensus sequence from 34 MAGs and is thus an average genome for '*M. sinica*'. From the outermost to the innermost ring: scale marks of the genome; protein-coding genes on the forward strand; protein-coding genes on the reverse strand; tRNA (black) and rRNA (red) genes on the forward strand; tRNA (black) and rRNA (red) genes on the reverse strand; GC content; GC skew. Protein-coding genes are color coded according to their COG categories.



Supplementary Figure 6: Dynamics of dissolved oxygen in the isotope labelling incubation system. Only nM range of dissolved oxygen (< 10 nM) was detected, indicating an intracellularly oxygenic process performed by '*M. sinica*' as previously reported[16].



Supplementary Figure 7: Representative images of cell sorting showing that before (a, c) and after (b, d) a single cell of '*M. sinica*' was successfully collected. Sorting operations were repeated independently over fifty times with similar results.

Supplementary Tables

Supplementary Table 1. The chemical profile of the paddy soil in Liangzhu (unit: mmol/kg)

Depth/cm	Nitrite	Nitrate	Methane
5	0.005	0.172	0.021
10	0.003	0.106	0.053
15	0.003	0.049	0.079
20	0.003	0.046	0.083
30	0.003	0.027	0.103
40	0.002	0.021	0.110
50	0.002	0.023	0.093

Supplementary Table 2. Relative abundance (16S rRNA gene) of ANME-2d archaea and *Methylomirabilis* bacteria based on metagenomic and transcriptomic datasets.

	Metagenome		**Transcriptome**	
Day	**ANME-2d**	***Methylomirabilis***	**ANME-2d**	***Methylomirabilis***
290	9.38×10^{-6}	1.29×10^{-1}	4.99×10^{-7}	1.78×10^{-1}
340	5.25×10^{-4}	7.90×10^{-2}	1.13×10^{-5}	2.49×10^{-1}
360	3.09×10^{-7}	2.24×10^{-1}	0	3.25×10^{-1}
380	2.15×10^{-7}	3.41×10^{-1}	0	4.78×10^{-1}

Supplementary Table 3. Summary of metagenomic, metatranscriptomic and metaproteomic data analyzed in the current study **(as separate Excel document)**.

Supplementary Table 4. Summary of 34 high-quality Metagenome Assembled Genomes (MAGs) with completeness >90%, contamination <5%[17] **(as separate Excel document)**.

Supplementary Table 5. 16S rRNA gene identity analysis of '*Ca.* Methylomirabilis sinica' **(as separate Excel document)**.

Supplementary Table 6. Presence core pathway genes detected in '*Ca.* Methylomirabilis sinica' genome **(as separate Excel document)**.

Supplementary Table 7. Comparison of genomes of '*Ca.* Methylomirabilis sinica' and other *Methylomirabilis* species[16,18,19,20] **(as separate Excel document)**.

Supplementary Table 8. 16S rRNA gene amplicon sequencing used for examination of the purity of '*Ca.* Methylomirabilis sinica'. Sequencing was performed on a MiSeq platform using universal primer pair 515F and 907R[12] **(as separate Excel document)**.

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