***Supplementary Material***

**Metabolic inactivity and re-awakening of a nitrate reduction dependent iron(II)-oxidizing bacterium *Bacillus ferrooxidans***

Guo-Wei Zhou1,2, Xiao-Ru Yang1,\*, Regin Rønn3, Jian-Qiang Su1, Li Cui1, Bang-Xiao Zheng4, and Yong-Guan Zhu1,2,5

*1Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China*

*2State Key Lab of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China*

*3Department of Biology, University of Copenhagen, Copenhagen Ø, Denmark*

*4Falculty of Biological and Environmental Sciences, University of Helsinki, Lahti, Finland,*

*5College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing, China.*

\* Corresponding Author

Phone: (+86) 592 6190997; Fax: (+86) 592 6190977;

E-mail: xryang@iue.ac.cn

Number of figures: 6.

Number of tables: 4.

1. **Supplementary materials and methods**

**PMA incubation experiments.**

In order to investigate the role of PMA in distinguishing the dead cells from living cells, five experimental treatments were set, including *Bacillus ferrooxidans* PMA addition, *B. ferrooxidans* no PMA addition, *B. ferrooxidans* (cell fragment) PMA addition, *Enterobacter* sp. (TCD1-1) PMA addition, and TCD1-1 no PMA addition (TCD1-1 was a strain isolated from a paddy soil in our lab and belonged to the genus *Enterobacter*).

For *B. ferrooxidans* PMA addition and TCD1-1 PMA addition, before PMA incubation, 650 μl (*B. ferrooxidans*; OD = 0.950) and 700 μl (TCD1-1; OD = 0.723) of cell suspension cultivated overnight (30 °C, R2A culture) were incubated with PMA (final concentration of PMA was 30 µmol L-1 in the mixture), respectively. Then, the following operation was consistent with the method describe previously.

*B. ferrooxidans* no PMA addition and TCD1-1 no PMA addition represented the treatments without PMA incubation. 650 μl (*B. ferrooxidans*; OD = 0.950) and 700 μl (TCD1-1; OD = 0.723) of cell suspension were collected by centrifuging (14000 g, 10 min), respectively.

For *B. ferrooxidans* (cell fragment) PMA addition, 650 μl (*B. ferrooxidans*; OD = 1.350) of cell was fragmented using homogenous cruder (6.0 m s-1, 40 s; FastPrep®-24, MP, America), and the cell suspension was incubated with PMA (final concentration of PMA was 30 µmol L-1 in the mixture) as described above.

**Measurement of 13CO2 assimilation in cells**

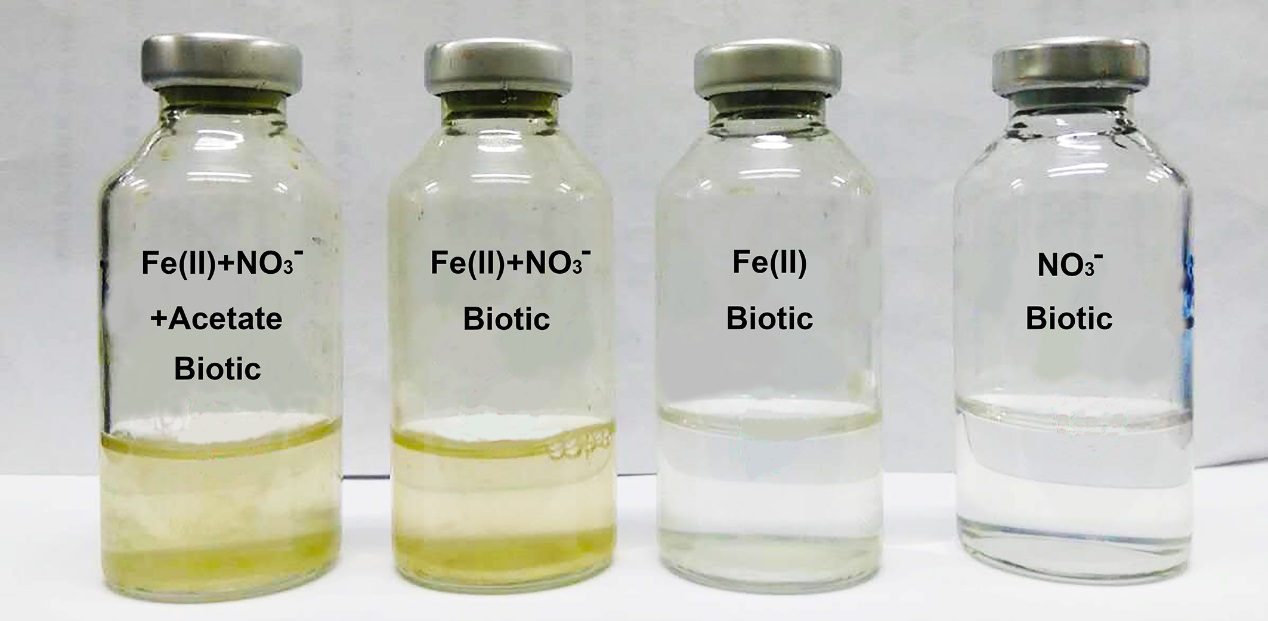
Strain *B. ferrooxidans* was cultivated according to the method described before with replacing NaH12CO3 with NaH13CO3 (13C 99%, [Cambridge Isotope Laboratories](https://www.isotope.com/)) during the incubation. After 120-h incubation, cells of strain *B. ferrooxidans* in NaH12CO3 and NaH13CO3 added FeOB media were harvested (8000 g, 10 min), washed three times with anoxic distilled water, then air-dried cells were used for Raman spectroscopy and freeze-dried samples were used for measurement of 13CO2 assimilation by GC-isotope ratio mass spectrometry (Thermo Finnigan Delta V Advantage, Bremen, Germany) ([Conrad et al. 2000](#_ENREF_2); [Zhou et al. 2017](#_ENREF_7)).

**Measurement of N2O, N2, NO2-, NH4+ and SOD activity**

Headspace N2O and N2 were determined by gas chromatography with a robotized incubation system (Agilent 7890, Santa Clara, CA, US) ([Molstad et al. 2007](#_ENREF_5)). A aliquot of 1 mL liquid sample was collected for analysis of NO2- and NH4+ in the anaerobic box. After filtrated through 0.22 μm filter, the concentrations were detected by chromatography (Dionex ICS-3000 system, Diones, Sunnyvale, CA, USA) ([Zhou et al. 2019](#_ENREF_8)). Cells were collected from R2A and FeOM after 12 hours of cultivation at 30 °C in the anaerobic, and SOD activity were analyzed using the xanthine–xanthine oxidase-nitroblue tetrazolium method ([Miyatake and Iwabuchi 2005](#_ENREF_4)).

1. **Supplementary Figures and Tables**
   1. **Supplementary Figures**

**Figure S1.** Images of *B. ferrooxidans* (YT-3) cultured on the Fe(II)-oxidizing and R2A plates at 30 °C in the anaerobic box. The “Abiotic FeO plate” represented the Fe(II)-oxidizing agar plate and was used as a control setup on which no cells were inoculated.



**Figure S2.** Images of *B. ferrooxidans* cultured in the different medium at 30 °C in the anaerobic box. Fe(II) + NO3- + Acetate represented the medium contained ferrous iron, nitrate and acetate. Fe(II) + NO3- represented the medium contained ferrous iron and nitrate. Fe(II) represented the medium contained ferrous iron. NO3- represented the medium contained NaNO3.



**Figure S3.** Images of *B. ferrooxidans* cultured in the Fe(II)-oxidizing medium inoculated with different densities of cells at 30 °C in the anaerobic box.

**Figure S4.** Kinetics of ferrous iron (A), nitrate reduction (B) and acetate (C) in the incubation of different medium. Fe(II) + NO3- + Acetate represented the medium contained ferrous iron, nitrate and acetate. Fe(II) + NO3- represented the medium contained ferrous iron and nitrate. The error bars represent standard deviations of three replications.

**Figure S5.** Raman spectra of *B. ferrooxidans* cells cultured in FeOB media added with NaH12CO3 (12C) and NaH13CO3 (13C). 745, 1130, 1318 and 1587 cm-1 were characteristic bands of cytochrome c. The shade of light blue represented the characteristic bands of phenylalanine. Shift of band from 1004 cm-1 to 972 cm-1 was observed in these two setups.



**Figure S6.** Gene copy numbers of bacterial 16S *rRNA* in six setups (*B. ferrooxidans* PMA addition, *B. ferrooxidans* no PMA addition, *B. ferrooxidans* (cell fragment) PMA addition, TCD1-1 PMA addition and TCD1-1 no PMA addition).

1. **Supplementary Tables**

**Table S1.** Primers and qPCR processes used in this study.

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| Genes | Primers  (sequences 5’-3’) | | qPCR processes | References | length |
| *narG* | *NarG1960f* | TAYGTSGGSCARGARAA | 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 40 s at 58°C and 40 s at 72°C. | ([Zhang et al. 2014](#_ENREF_6)) | 420 bp |
| *NarG2650r* | TTYTCRTACCABGTAGC |
| *nasA* | nasA964  nasA1735 | CARCCNAAYGCNATGGG  ATNGTRTGCCAYTGRTC | 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 40 s at 60°C and 35 s at 72°C. | ([Allen et al. 2001](#_ENREF_1)) | 756 bp |
| *nosZ* | *nosZ*-F  *nosZ*-1622R | CGYTGTTCMTCGACAGCCAG  CGSACCTTSTTGCCSTYGCG | 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C and 34 s at 72°C. | ([Kandeler et al. 2006](#_ENREF_3)) | 435 bp |

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| **Table S2.** Concentrations of N2O and N2 produced from setups in the FeOM after 120-hour incubation.   |  |  |  | | --- | --- | --- | | Setups | N2O (mmol L-1) | N2 (mmol L-1) | | Control (Sterile) | ND | ND | | *B. ferrooxidans* (1×) | 0.14 ± 0.021 | 0.12 ± 0.052 |   ND: not detected.  **Table S3.** Values of 13C/total carbon in cells of strain *B. ferroxidans* cultivated in FeOB media added with NaH12CO3 and NaH13CO3.   |  |  | | --- | --- | | Setups | Value | | Cells in NaH12CO3-added medium | 0.0107 ± 0.0000100 | | Cells in NaH13CO3-added medium | 0.0134 ± 0.0000770\* |   **\*** indicate significant difference between the treatments at *P* < 0.05 using independent samples t-test. |
| **Table S4.** SOD activity of cells in two setups.   |  |  | | --- | --- | | Setups | SOD activity (U mg-1 protein) | | Cells from R2A | 13.22 ± 1.68 | | Cells from ANDFO | 38.75 ± 5.45\* | |

\*indicate significant difference between the treatments at *P* < 0.05 using independent samples t-test.

1. **Supplementary References**

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