



OPEN

SUBJECT AREAS:

ENVIRONMENTAL
BIOTECHNOLOGY

WATER MICROBIOLOGY

Received
28 July 2014Accepted
29 December 2014Published
23 January 2015

Correspondence and requests for materials should be addressed to W.-W.L. (wwli@ustc.edu.cn) or H.-Q.Y. (hgyu@ustc.edu.cn)

* These authors contributed equally to this work.

Roles of 3,3',4',5-tetrachlorosalicylanilide in regulating extracellular electron transfer of *Shewanella oneidensis* MR-1

Yong-Peng Wang^{1,2*}, Sheng-Song Yu^{1*}, Hai-Ling Zhang^{1,2}, Wen-Wei Li^{1,3}, Yuan-Yuan Cheng¹ & Han-Qing Yu¹

¹Department of Chemistry, University of Science & Technology of China, Hefei, 230026, China, ²China Academy of Engineering Physics, P. O. Box 919, Mianyang 621900, China, ³Collaborative Innovation Center of Suzhou Nano Science and Technology, University of Science & Technology of China, Hefei, 230026, China.

Microbial extracellular electron transfer (EET) is critically involved in many pollutant conversion processes in both natural environment and engineered bioelectrochemical systems (BES), but typically with limited efficiency and poor controllability. In this study, we discover an important role of uncouplers in affecting the microbial energy metabolism and EET. Dose of lower-concentration 3,3',4',5-tetrachlorosalicylanilide (TCS) in the anolyte promoted the current generation and substrate degradation of an MFC inoculated with *Shewanella oneidensis* MR-1. However, higher TCS dosage caused obvious microbial inhibition. Our results suggest a previously unknown role of uncouplers in regulating the microbial EET. In addition, the underlying mechanisms of such processes are investigated. This work broadens our view about the EET behaviors of microorganisms in real water environment where uncouplers are usually present, and suggests a possible new approach to regulate microbial EET in BES.

Microorganisms can gain energy from the metabolism-associated electron transfer processes for proliferation and maintenance¹⁻³. In particular, some microbial species have developed the ability to respire external electron acceptors and exert non-negligible influences on processes from geochemical metal cycling to water environmental bioremediation and energy production⁴⁻⁷. Thus, extracellular electron transfer (EET), as a critical foundation of all these processes, needs more attention and careful manipulation^{2,8}. However, such processes in real environment scenario are still poorly understood^{9,10}. Especially, given the usually low EET efficiency and controllability in actual systems¹¹, effective approaches for regulating the microbial EET process are needed.

In metabolism, microbes invest a considerable fraction of generated energy to sustain cell growth and metabolic activity. Under aerobic conditions, the substrate oxidation and electron transfer create a proton motive force (PMF) across the intracellular cytoplasm membrane, which drives the ATP formation via phosphorylation of ADP. Thus, the energy metabolism and electron transport processes are normally coupled by the conservation of free energy in the form of ATP¹². However, this coupling can be disrupted or even completely broken by uncouplers to short-circuit the PMF¹²⁻¹⁴. As a result, the growth of aerobic cells would decrease in the presence of uncoupler due to restrained ATP production and reduced energy for biomass synthesis¹⁵⁻¹⁷. However, can the energy metabolism and EET of bacteria under anaerobic conditions also be affected by uncoupler? Even more, could this be adapted as a possible way for microbial EET manipulation? These questions remain unsolved so far.

Herein, we conducted a first investigation into this process by using *Shewanella oneidensis* MR-1 as a model bacterium and 3,3',4',5-tetrachlorosalicylanilide (TCS) as a metabolic uncoupler. TCS has been widely adopted as an environmentally-benign uncoupler to reduce yield of activated sludge^{18,19}. Our results show that TCS could also significantly affect the anaerobic metabolism and electron transfer processes. In light of the ubiquity of uncouplers in natural environment, our work implies that the EET processes in natural environments and various engineered systems might need to be reexamined. Moreover, this work might also open up a useful new approach to engineer microbial EET and bioelectrochemical processes.



Results

Electricity generation and EET performances of MFCs. MFC tests were conducted to investigate the effects of TCS on the EET of *S. oneidensis* MR-1. A moderate TCS concentration of 50 $\mu\text{g/L}$ was adopted. As shown in Fig. 1, while no voltage was detected in all the abiotic and dead-cell controls, considerable power generation was observed in all the inoculated MFC, suggesting that the electron generation relies critically on the activities of the inoculated *Shewanella* cells. Notably, the TCS effects on electricity generation were concentration dependent. Addition of 50 $\mu\text{g/L}$ was found to significantly increase the output voltage compared to the TCS-free control, while 400 $\mu\text{g/L}$ led to obvious suppression.

This concentration-dependent impacts of TCS could be more clearly seen from the total recovered charge (Fig. 2). TCS of 50 $\mu\text{g/L}$ lead to the highest quantity of recovered electricity in the MFC, which was over two-fold higher than the TCS-free control. However, a distinct drop of the electricity generation was observed when further increasing the TCS dosage. The recovered electricity at 400 $\mu\text{g/L}$ TCS was even below the control. These results suggest that lower concentration of TCS could promote the microbial EET while a higher concentration may lead to inhibition.

Lactate degradation. The lactate consumption rates, which indirectly reflect the overall microbial activity, also showed a similar trend in response to TCS addition, with the highest removal obtained at 50 $\mu\text{g/L}$ TCS while the lowest achieved at 400 $\mu\text{g/L}$ (Fig. 3). The statistical analysis of the three sets of data showed F and P values of 8.67963 and 0.01695 respectively, suggesting that the lactate consumption rates under the tested conditions are significantly different ($p < 0.05$). Notably, no significant differences in the biomass growth were observed regardless of the different TCS strength (data not shown). Thus, the promotion of lactate consumption at lower TCS dosage was likely associated with a shift of substrate metabolic pathway, as detailed below.

Notably, similar concentration-dependent impacts of TCS (i.e., promoting EET at lower concentrations but inhibiting at higher concentrations) were also observed for several other EABs (data not shown), indicating that TCS could serve as an effective regulator to tune the microbial EET for a wide spectrum of bacteria.

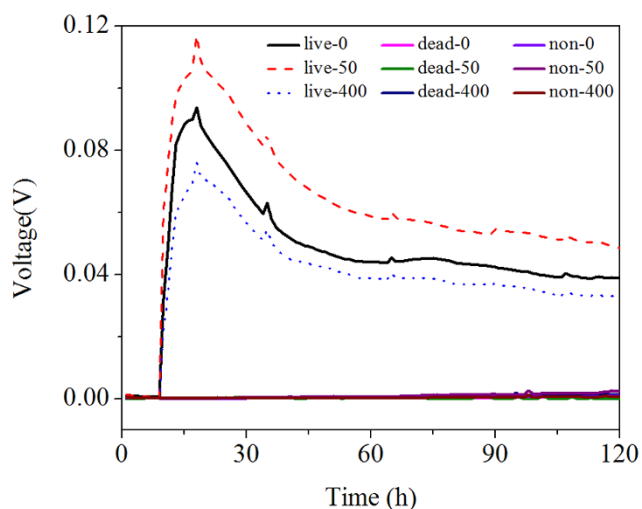


Figure 1 | Voltages of MFCs under different inoculum and TCS dosage conditions. Here, live, dead and non means inoculating with live cells, dead cells and without cells; 0, 50, and 100 refers to the values of added TCS concentrations (in $\mu\text{g/L}$).

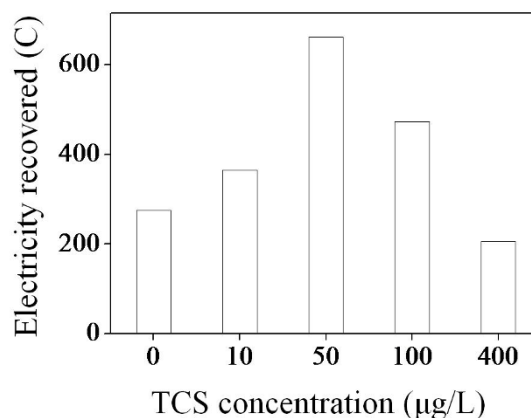


Figure 2 | Recovered electricity in the MFCs at different TCS concentrations.

Discussion

The above results demonstrate a concentration-dependent effect of TCS on microbial EET and metabolism. The underlying mechanisms of EET regulation by uncouplers must be different from that by electron mediator, as the latter solely shuttle electrons between bacteria and extracellular electron acceptors^{20–22}.

Fig. 4a depicts the dominant pathways of electron transfer and energy metabolism within *Shewanella* cells under normal conditions (without uncoupler addition). Firstly, the electrons derived from substrate oxidation are transferred to electron-transporting chain (ETC) proteins, from which a part of the electrons are further delivered outside. The ETC also acts as a proton pump to drive protons out by using the energy carried in electrons. As a consequence, accompanied with the electron transfer process, a PMF is built up across the membrane. Such a PMF plays a critical role in ATP synthesis. A part of the protons further diffuse outside the cells, while another part are driven back into the cells through the ATP synthases under the PMF, during which process the energy carried by protons are converted to chemical energy in ATP bonds²³. Therefore, under this circumstance the microbial electron transfer is coupled with the ATP synthesis process. This kind of metabolic pathway, which is also termed as oxidative phosphorylation, presents a highly efficient way for bacteria to obtain and conserve energy.

However, such a coupling was significantly weakened by TCS. Fig. 4b shows the possible patterns of substrate metabolism, electron transport and ATP synthesis in the presence of TCS. Generally, TCS as an uncoupler may affect the energy metabolism and activity of microbial cells by three mechanisms: 1) directly uncoupling the metabolism through dispersing the PMF and hence reducing the ATP synthesis for energy production^{19,24}; 2) inducing more EPS secretion, especially proteins, so as to resist the toxicity of TCS²⁵.

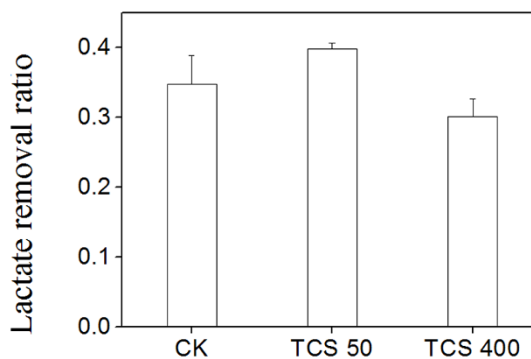


Figure 3 | Lactate consumption with different TCS dosage after 72-h incubation.

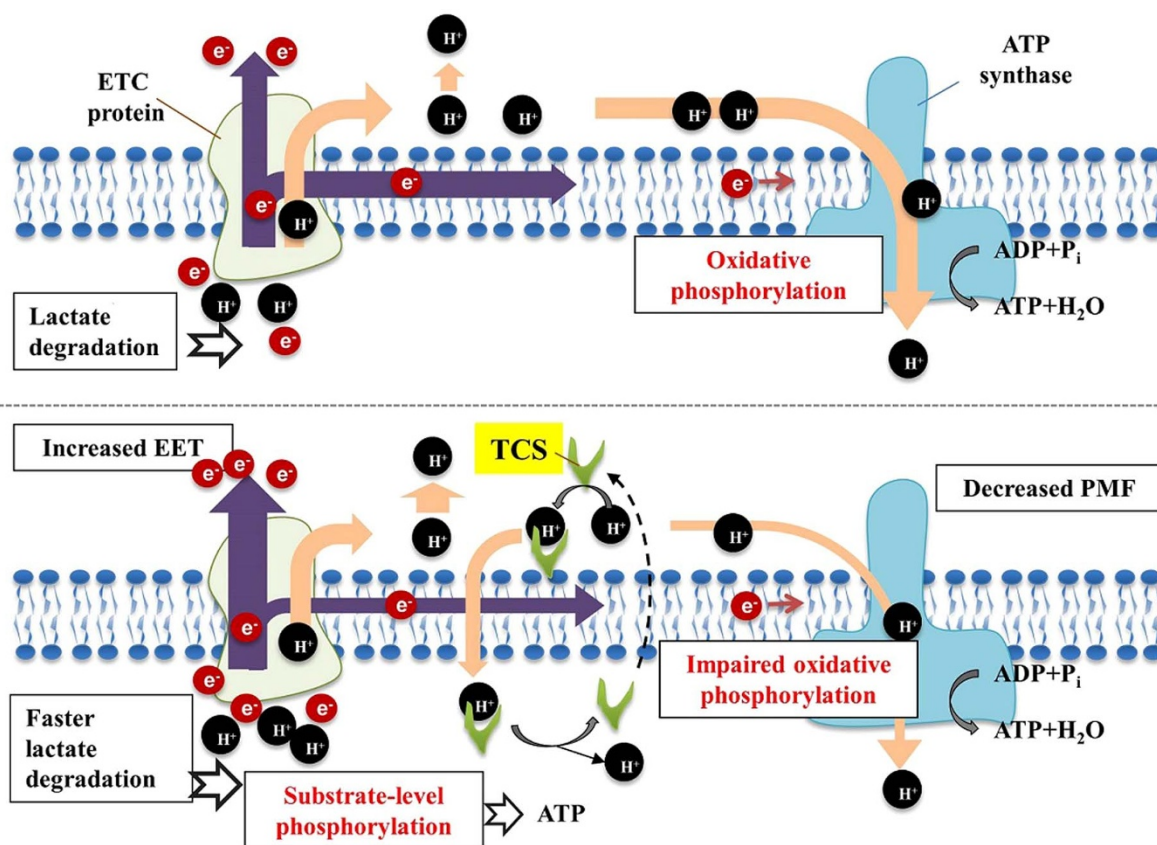


Figure 4 | Pathways of cross-membrane electron transfer, proton transport, and energy metabolism of electrochemical active microorganisms (a) under normal conditions; and (b) with TCS addition. The orange and purple arrows represent the move directions of protons and electrons respectively.

Similar phenomenon was also observed in the presence of high free chlorine²⁶. The increased secretion of extracellular polymeric substances consumes extra energy and further reduces available energy for microbial growth; 3) TCS at higher concentrations may cause microbial inhibition and even trigger cell lysis^{27,28}. These mechanisms may also apply in our system depending on the TCS dosage and environmental conditions. In addition, several other processes might be intrigued by TCS, thereby leading to different substrate metabolism and electron transfer behaviors. Firstly, TCS as a proton carrier could introduce a new pathway of proton transfer across the membrane and weaken the PMF, which favors a more efficient pumping of electrons and protons cross the membrane due to a lower electrochemical resistance. This could explain the increased EET and electricity generation of the MFCs with lower TCS concentration. Interestingly, the TCS led to suppressed ATP synthesis via the oxidative phosphorylation route though, no significant decrease in cell growth was observed in our study. Instead, the lactate uptake of *S. oneidensis* MR1 was slightly stimulated by the 50 $\mu\text{g/L}$ of TCS. This should be associated with a shift of the metabolic pathway for *S. oneidensis* MR1 under anaerobic conditions. Due to a suppressed oxidative phosphorylation in the presence uncoupler, the cells are forced to metabolize substrate by a fermentative pathway. The latter process also generates small amount of ATP but is independent of PMF²⁹. Thus, in order to compensate for the energy deficiency, the cells may aggressively uptake more substrate due to energy hungry effects^{30,31}. This may, to certain degree, compensate for the decreased biomass synthesis caused by metabolic uncoupling. On the other hand, an over high TCS level (400 $\mu\text{g/L}$ in this case) may severely impair the ATP-synthesis and enzyme activity, thereby suppressing the microbial activity for substrate uptake and electricity generation. This TCS strength is unlikely to induce cell lysis in the short-term operation according to pervious studies^{18,25}, but the long-term

impacts are yet to be investigated. Moreover, many of the proposed mechanisms here still need to be validated in future studies.

In summary, this study report a new discovery that microbial EET process could be affected by TCS. It expands our knowledge on the EET behaviors of microorganism in real environments whereby uncouplers like TCS are usually present, and opens up a new possibility for manipulating microbial EET and promoting microbial-catalysis associated applications.

Methods

Inocula and cultivation conditions. The frozen stocks of *Shewanella oneidensis* MR-1 was provided by Kenneth H. Nealson from the University of Southern California and Xiao Xiang from Shanghai Jiao Tong University. Before test, the strain was inoculated from frozen stocks and grown in LB medium at 30°C until the exponential phase.

A sterile sodium lactate minimal salt medium was used as the cultivation medium and was fed into the anodic chamber. The medium contained (in per liter): 3.36 g sodium lactate, 5.85 g NaCl, 11.91 g Hepes, 0.3 g NaOH, 1.498 g NH_4Cl , 0.097 g KCl, 0.67 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, plus 0.4 ml of a trace mineral stock solution (containing per liter: 1.5 g NTA ($\text{C}_6\text{H}_9\text{NO}_6$), 30 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 10 g NaCl, 1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.3 g ZnCl_2 , 0.1 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 g $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.1 g H_3BO_3 , 0.25 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$). After autoclaving, the sterilized medium was added with 0.4 ml of filter-sterilized amino acid solution (containing per liter: 2 g L-glutamic acid, 2 g L-arginine, 2 g DL-serine) and 0.4 ml of filter-sterilized vitamin solution (containing per liter: 2.0 g biotin, 2.0 g folic acid, 10.0 g pyridoxine HCl, 5.0 g riboflavin, 5.0 g thiamine, 5.0 g nicotinic acid, 5.0 g pantothenic acid, 0.1 g cyanocobalamin, 5.0 g p-aminobenzoic acid, 5.0 g thioctic acid).

TCS, a protonophore widely present in the environment and commonly used proton shuttler, was selected as a model uncoupler for the tests. All chemicals used in this study were of analytical grade, and the solutions were prepared with ultrapure water (Millipore Co., USA).

MFC experiments. Dual-chamber MFCs with a 110-mL anodic chamber were used to validate the electricity generation capability of the tested bacteria³². Carbon felt (Beijing Sanye Carbon Co., China) with a projected surface area of 8 cm^2 was used as both the anode and cathode materials. The electrodes were connected via an external



circuit with a 1000-Ω resistance. A proton exchange membrane (GEFC-10N, GEFC Co., China) was used as the separator. The catholyte consisted of 50 mM potassium ferricyanide in phosphate buffer solution (50 mM, pH 7.0). Prior to the experiment, both the anode and cathode chambers were flushed with ultrapure nitrogen gas to ensure an anaerobic atmosphere. The anode chamber was inoculated with *S. oneidensis* MR-1. After adding 100 mL of sterile mineral salt medium containing sodium lactate as carbon source, a pre-determined concentration of TCS was dosed into the anode chambers of MFCs. Three other MFCs without inoculation were used as the abiotic control, while another three MFCs inoculated with dead cells were used as the dead-cells control. The MFCs were operated in a batch mode at $25 \pm 1^\circ\text{C}$, and all tests were conducted in triplicate.

The voltage across the external resistance was continuously recorded by using a data acquisition/switch unit (34970A, Agilent Inc., USA) connected to a computer.

Analysis. Lactate concentration was measured using a high performance liquid chromatograph (HPLC, 1101, Agilent Inc., USA) equipped with an ultraviolet (at 210 nm) detector and a C18 reversed phase chromatographic column. The mobile phase consisted of 97.5% H_3PO_4 solution (1 mL 99.5% HPLC-grade H_3PO_4 in 1 L deionization water, pH 3.0) and 2.5% acetonitrile solution (HPLC grade).

- Lovley, D. R. The microbe electric: conversion of organic matter to electricity. *Curr. Opin. Biotechnol.* **19**, 564–571 (2008).
- Logan, B. E. Exoelectrogenic bacteria that power microbial fuel cells. *Nat. Rev. Microbiol.* **7**, 375–381 (2009).
- Liu, X. W., Li, W. W. & Yu, H. Q. Cathodic catalysts in bioelectrochemical systems for energy recovery from wastewater. *Chem. Soc. Rev.* **43**, 7718–7745 (2014).
- Li, W. W. & Yu, H. Q. From wastewater to bioenergy and biochemicals via two-stage bioconversion processes: A future paradigm. *Biotechnol. Adv.* **29**, 972–982 (2011).
- Rabaey, K. & Verstraete, W. Microbial fuel cells: novel biotechnology for energy generation. *Trends Biotechnol.* **23**, 291–298 (2005).
- Mu, Y., Rabaey, K., Rozendal, R. A., Yuan, Z. & Keller, J. Decolorization of azo dyes in bioelectrochemical systems. *Environ. Sci. Technol.* **43**, 5137–5143 (2009).
- Mu, Y., Rozendal, R. A., Rabaey, K. & Keller, J. Nitrobenzene removal in bioelectrochemical systems. *Environ. Sci. Technol.* **43**, 8690–8695 (2009).
- Wu, C. *et al.* Electron acceptor dependence of electron shuttle secretion and extracellular electron transfer by *Shewanella oneidensis* MR-1. *Bioresour. Technol.* **136**, 711–714 (2013).
- Rabaey, K., Angenent, L., Schroder, U. & Keller, J. Bioelectrochemical systems: from extracellular electron transfer to biotechnological application. (IWA Publishing London; 2010).
- Freguia, S., Tsujimura, S. & Kano, K. Electron transfer pathways in microbial oxygen biocathodes. *Electrochim. Acta* **55**, 813–818 (2010).
- Rabaey, K. *et al.* Microbial ecology meets electrochemistry: electricity-driven and driving communities. *ISME J.* **1**, 9–18 (2007).
- Cook, G. M. & Russell, J. B. Energy-spilling reactions of *Streptococcus-bovis* and resistance of its membrane to proton conductance. *Appl. Environ. Microbiol.* **60**, 1942–1948 (1994).
- Mayhew, M. & Stephenson, T. Low biomass yield activated sludge: A review. *Environ. Technol.* **18**, 883–892 (1997).
- Liu, Y. Energy uncoupling in microbial growth under substrate-sufficient conditions. *Appl. Microbiol. Biotechnol.* **49**, 500–505 (1998).
- Liu, Y. Effect of chemical uncoupler on the observed growth yield in batch culture of activated sludge. *Water Res.* **34**, 2025–2030 (2000).
- Russell, J. B. The energy spilling reactions of bacteria and other organisms. *J. Mol. Microbiol. Biotechnol.* **13**, 1–11 (2007).
- Saini, G. & Wood, B. D. Metabolic uncoupling of *Shewanella oneidensis* MR-1, under the influence of excess substrate and 3,3',4',5-tetrachlorosalicylanilide (TCS). *Biotechnol. Bioeng.* **99**, 1352–1360 (2008).
- Li, Y. *et al.* SMP production by activated sludge in the presence of a metabolic uncoupler, 3,3',4',5-tetrachlorosalicylanilide (TCS). *Appl. Microbiol. Biotechnol.* **95**, 1313–1321 (2012).
- Chen, G.-H., Mo, H.-K. & Liu, Y. Utilization of a metabolic uncoupler, 3,3',4',5-tetrachlorosalicylanilide (TCS) to reduce sludge growth in activated sludge culture. *Water Res.* **36**, 2077–2083 (2002).
- Park, D. H., Kim, S. K., Shin, I. H. & Jeong, Y. J. Electricity production in biofuel cell using modified graphite electrode with Neutral Red. *Biotechnol. Lett.* **22**, 1301–1304 (2000).
- Chen, J. J. *et al.* Manipulation of microbial extracellular electron transfer by changing molecular structure of phenazine-type redox mediators. *Environ. Sci. Technol.* **47**, 1033–1039 (2013).
- Marsili, E. *et al.* *Shewanella* Secretes flavins that mediate extracellular electron transfer. *Proc. Nat. Acad. Sci.* **105**, 3968–3973 (2008).
- Garrett, R. H. & Grisham, C. M. *Biochemistry*, Edn. third. (Thomson Brooks/Cole, Belmont, CA; 2005).
- Miller, E., Wohlfarth, G. & Diekert, G. Studies on tetrachloroethene respiration in *Dehalospirillum multivorans*. *Arch. Microbiol.* **166**, 379–387 (1996).
- Feng, X. C. *et al.* Possible causes of excess sludge reduction adding metabolic uncoupler, 3,3',4',5-tetrachlorosalicylanilide (TCS), in sequence batch reactors. *Bioresour. Technol.* **173**, 96–103 (2014).
- Leriche, V., Briandet, R. & Carpentier, B. Ecology of mixed biofilms subjected daily to a chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect of one species to another. *Environ. Microbiol.* **5**, 64–71 (2003).
- Tian, Y., Zhang, J., Wu, D., Li, Z. & Cui, Y. Distribution variation of a metabolic uncoupler, 2,6-dichlorophenol (2,6-DCP) in long-term sludge culture and their effects on sludge reduction and biological inhibition. *Water Res.* **47**, 279–288 (2013).
- Ye, F. X., Shen, D. S. & Li, Y. Reduction in excess sludge production by addition of chemical uncouplers in activated sludge batch cultures. *J. Appl. Microbiol.* **95**, 781–786 (2003).
- Pinchuk, G. E. *et al.* Pyruvate and lactate metabolism by *Shewanella oneidensis* MR-1 under fermentation, oxygen limitation, and fumarate respiration conditions. *Appl. Environ. Microbiol.* **77**, 8234–8240 (2011).
- Heiden, M. G. V., Cantley, L. C. & Thompson, C. B. Understanding the Warburg Effect: The metabolic requirements of cell proliferation. *Science* **324**, 1029–1033 (2009).
- Heiden, M. G. V. *et al.* Evidence for an alternative glycolytic pathway in rapidly proliferating cells. *Science* **329**, 1492–1499 (2010).
- Li, W. W. *et al.* Impact of a static magnetic field on the electricity production of *Shewanella*-inoculated microbial fuel cells. *Biosens. Bioelectron.* **26**, 3987–3992 (2011).

Acknowledgments

The authors wish to thank the NSFC (51278479 and 51129803), and the Program for Changjiang Scholars and Innovative Research Team in University, China for the partial support of this study. In addition, we would also like to thank Prof. K. H. Neelson from the University of Southern California for his kind provision of *Shewanella oneidensis* MR-1 wild type and its mutant strains.

Author contributions

Y.P.W., S.S.Y. and H.Q.Y. designed the experiments; Y.P.W., S.S.Y., H.L.Z., W.W.L., Y.Y.C. conducted the experiments; Y.P.W., W.W.L. and H.Q.Y. contributed to the planning and coordination of the project and writing of the paper; All authors contributed to discussion about the results and the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Wang, Y.-P. *et al.* Roles of 3,3',4',5-tetrachlorosalicylanilide in regulating extracellular electron transfer of *Shewanella oneidensis* MR-1. *Sci. Rep.* **5**, 7991; DOI:10.1038/srep07991 (2015).



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>