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As featured in:



See Hashimoto, Nakamura *et al., Energy Environ. Sci.*, 2014, **7**, 1357.





Energy & Environmental Science

COMMUNICATION



View Article Online View Journal | View Issue

Open Access Article. Published on 12 February 2014. Downloaded on 8/27/2025 5:58:06 AM.

Cite this: Energy Environ. Sci., 2014, 7, 1357

Received 8th November 2013 Accepted 13th January 2014

DOI: 10.1039/c3ee43674h

www.rsc.org/ees

Uptake of self-secreted flavins as bound cofactors for extracellular electron transfer in *Geobacter* species[†]

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Geobacter species are among the most efficient current-producing bacterial species, yet their electron-transfer mechanisms have been scarcely investigated at the molecular level. Here, we provide evidence that *Geobacter* cells secrete and utilize riboflavin as a bound-cofactor in outer-membrane *c*-type cytochromes. This finding highlights the potential roles of riboflavin as a major electron carrier in current production.

Electron transfer from cell metabolic systems to exterior solid substrates, termed extracellular electron transfer (EET), is an intriguing aspect of microbial respiration.^{1,2} In anaerobic environments, particularly biofilms, EET is a terminal step of catabolism and is involved in redox sensing³ and intercellular⁴/ interspecies⁵ energy transfer. It is also a fundamental process in microbial communities involved in energy production (*e.g.*, microbial fuel cells),⁶ bioremediation of waste waters and contaminated sediments,⁷ and anaerobic pipeline corrosion.⁸

Over the last decade, several mechanisms for microbial EET have been proposed, including indirect electron transfer *via* redox-active organic electron shuttles^{9,10} and direct electron transfer by *c*-type cytochromes (*c*-Cyts) located in the outer membrane (OM)¹¹ or on nanometer-scale bacterial filaments.^{12,13} In indirect EET, microbial cells are able to perform electron transfer without the necessity of direct contact with solid-phase electron acceptors. Thereby, as the surface area of the solids is limited, EET *via* self-secreted, naturally occurring or artificially supplemented soluble electron shuttles appears to

Broader context

Geobacter sulfurreducens is an iron-reducing bacterium that has a significant content of c-type cytochromes (c-Cyts) in an outer-cell membrane (OM) and bacterial filaments, drawing keen attention as a model microorganism for the research of microbial fuel cells and bioremediation technology. Concerned with the ability of Geobacter to transport electrons to electrodes in microbial fuel cells, processes termed extracellular electron transport (EET), a great deal of research has been focused on identifying the molecular mechanisms behind the bacterial current generation. Herein, we report the first evidence that Geobacter utilizes selfsecreted flavins as a redox cofactor in OM c-Cyts. Using a highly sensitive voltammetry technique, the key redox signal for the Geobacter EET was identified in vivo. Experiments using a mutant unable to produce OM c-Cyts, together with the spectroscopic and LC-MS analyses, revealed that the noble redox signal is assigned to the bound riboflavin associated with OM c-Cyts containing reduced hemes. As the heme redox state reflects a balance between the electron input from respiration and the output by EET, the present study not only significantly contributes to the molecular understanding of EET, but also highlights the microbial capability of utilizing self-secreted riboflavin as a regulator for intracellular redox homeostasis.

be a major pathway for electron transfer to solids.¹⁰ However, such soluble redox molecules are apparently not involved in the EET process of Geobacter sulfurreducens,¹⁴ which is the most efficient current-producing microorganism characterized to date. This conclusion was reached after it was shown that the exchange of supernatants to fresh medium in an electrochemical reactor did not impair current production by G. sulfurreducens as it did in other microorganisms.14 Recently, we reported that cell-secreted redox molecules, such as flavins, have a high affinity for OM c-Cyts with reduced hemes in Shewanella oneidensis MR-1.15 This flavin binding to OM c-Cyt scaffolds was found to facilitate a one-electron redox reaction *via* semiguinone, resulting in a 10^3 - to 10^5 -fold enhancement of the EET rate compared to free flavin.¹⁵ Based on this finding, we speculated that Geobacter cells might also use self-secreted redox molecules as redox cofactors to promote EET. If true, the excreted molecules should not act as a diffusion-based electron

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[†] Electronic supplementary information (ESI) available: Figures and methods. See DOI: 10.1039/c3ee43674h

shuttle in *G. sulfurreducens*, and thereby the supernatant exchange could not impair current production by *G. sulfurre-ducens*.¹⁴ This possibility is supported by the fact that, similar to *S. oneidensis* MR-1,¹⁶ *G. sulfurreducens* have genes for both flavin biosynthesis and secretion (Fig. S1†), although their functions have not been confirmed.

In this paper, we report that *G. sulfurreducens* secrete flavin that contributes to EET at the cell/electrode interface, highlighting the crucial role of flavin redox cycling for efficient EET in this species.

To directly examine the secretion of flavin by G. sulfurreducens, a supernatant solution of anaerobically grown cultures was subjected to spectroscopic measurements (Fig. 1). Geobacter cells were grown in a defined medium supplemented with acetate (20 mM) as an electron donor and fumarate (80 mM) as an electron acceptor. As shown in Fig. 1a and b, both the peak intensities of emission at 525 nm and excitation at 370 and 440 nm increased when the growth curve of cells was in a sigmoidal phase. The emission and excitation spectral peaks were identical to those observed in the spectra of riboflavin (RF) and flavin mononucleotide (FMN) solutions without cells (Fig. S2a and b⁺), suggesting that G. sulfurreducens cells secrete flavin species during growth. In support of these data, mass chromatography patterns of the cell culture supernatant measured in selective ion monitoring (SIM) mode at m/z 375.35 identified that RF was present at a concentration of 100 nM, although FMN at m/z 455.34 was under the detection limit (Fig. 1c and S3[†]). These findings provide evidence that G. sulfurreducens secrete RF at concentrations comparable to that observed in cultures of anaerobically grown S. oneidensis MR-1.17

The contribution of secreted RF for EET was examined by adding a concentrated RF solution during the electrochemical cultivation of *G. sulfurreducens*. Current production (I_c) was



We plotted $I_{-0.2}$ against I_c to quantify the contribution of the redox species at -0.2 V to EET. As shown in Fig. 2, I_c exhibited a positive correlation with $I_{-0.2}$, as a fitted line passed through the point of origin with a high correlation coefficient ($r^2 = 0.998$). Importantly, this trend was observed both before and after the addition of RF, and even after the addition of malonic acid as a metabolic inhibitor (Fig. 2d and S4†). These results confirm the assignment of the peak potential (E_p) at -0.2 V to the redox cycling of RF, and demonstrate that respiratory current generation by *G. sulfurreducens* is strongly coupled with the amount of electrochemically active RF. In addition, FMN exhibited a similar effect on the I_c and peak current to that seen with RF, but at a different E_p (Fig. S6†). Upon the addition of FMN, both the peak current at -0.175 V ($I_{-0.175}$) and I_c increased, and a



Fig. 1 Detection of flavins secreted by *G. sulfurreducens* during growth in anaerobic medium. Emission (a) and excitation (b) spectra of the cell culture supernatant after 0, 15, 25, 39, 48, 63, 72, 87 h of incubation. (c) Mass chromatography patterns of the cell culture supernatant after 87 h of anaerobic culture. RF and FMN were detected by selected ion monitoring mode at 375.35 and 455.34 *m/z*, respectively.



Fig. 2 Role of riboflavin (RF) in extracellular electron transfer (EET) by *G. sulfurreducens*. (a) Current (I_c) versus time (t) measurements for microbial current generation by *G. sulfurreducens* on an ITO electrode. Arrows indicate the time when differential pulse (DP) voltammetry was measured. (b) DP voltammograms measured at t = 6, 20, and 46 h in panel a. (c) Plot of I_c in panel a against the peak current (I_p) at peak potentials (E_p) of -0.2, -0.1 and 0 V. (d) Plot of I_c against I_p at an E_p of -0.2 V before and after the addition of 1 μ M RF and 100 mM malonic acid. The squares of the correlation coefficients were estimated by the addition of the point of origin to the obtained data.

fitted line for the plot of $I_{-0.175}$ and I_c passed through the point of origin (Fig. S6†), demonstrating the capability of *G. sulfurreducens* to utilize FMN as an efficient electron carrier. Together, these data provide evidence for the involvement of RF and/or FMN as important electron carriers at the interface between *Geobacter* cells and ITO electrodes.

The measured E_p in the DP voltammogram of RF and FMN solutions in the absence of cells significantly differ from those observed in Geobacter biofilms (Fig. S7[†]), suggesting that both flavins alter their redox properties, as is reported for flavins bound to OM c-Cyts in S. oneidensis MR-1.15 Assuming one of the c-Cyts located on bacterial filaments or embedded in the OM interacts with flavins as bound cofactors in G. sulfurreducens, insight into the location of c-Cyts that bind RF is important to determine the predominant EET pathway in this species. Fig. 3 shows scanning electron microscopy (SEM) images of G. sulfurreducens cells attached to the ITO electrode after 50 h of electrode cultivation. Neither filament-like assemblages nor multilayer biofilms were formed on the electrode surface, corresponding to a previous report where thick biofilms of G. sulfurreducens cells with Cyt-bound filaments required more than 4 days of electrochemical cultivation.18 Therefore, it appears that the EET process mediated by the redox cycling of flavins observed in Fig. 2 is the consequence of the activation of c-Cyts located primarily on the OM surface, as opposed to those located on the surface of conductive bacterial filaments.

To examine the specific interactions between flavins and OM *c*-Cyts, we used a mutant strain ($\Delta omcBEST$) of *G. sulfurreducens* lacking OmcB, OmcE, OmcS, and OmcT, which are the major multi-heme c-Cyts localized on the OM of Geobacter.19 When cultured at +0.4 V in the presence of 10 mM acetate, current production by the $\Delta omcBEST$ strain was highly impaired (Fig. S8[†]). In addition, the peak position and I_p of RF displayed a negative shift of 75 mV and 20-fold decrease, respectively, compared to those of the wild-type (WT) strain (Fig. 4a and S9[†]). Under FMN-supplemented conditions, the E_p of $\Delta omcBEST$ also exhibited a 50 mV negative shift in the DP voltammogram and a large I_p decrease compared to that of WT (Fig. S10[†]). These significant effects on $E_{\rm p}$ observed for the $\Delta omcBEST$ strain demonstrate that both FMN and RF associate with OmcB, OmcE, OmcS, or OmcT c-Cyt proteins to enhance the rate of EET in WT G. sulfurreducens cells. Such specific association of RF



Fig. 3 SEM images of the ITO electrode surface after 50 h of electrochemical cultivation of *G. sulfurreducens* at 0.4 V (vs. SHE) in the presence of 10 mM acetate. Filament-like structures were hardly detected.



Fig. 4 (a) Base-line subtracted differential pulse (DP) voltammograms of riboflavin (RF) for cell-free RF 10 μ M solution (blue) and for *G. sul-furreducens* wild type (WT) (black) and $\Delta omcBEST$ strain (red) in the presence of 2 μ M RF. (b) Schematic model illustration of interactions between RF and OM *c*-Cyts in extracellular electron transport (EET) processes. Free RF (yellow dots) scarcely involves in EET ($E_p = -0.27$ V vs. SHE), but bound RF associated with OM *c*-Cyts, omcBEST proteins (blue dots), works as a major EET pathway ($E_p = -0.2$ V). Although RF still binds to the remaining OM *c*-Cyts in the absence of omcBEST (E_p at -0.12 V) (a pale yellow dot), a decrease in the amount of bound RF results in a significantly lower current production compared with WT.

with OM c-Cyts of G. sulfurreducens cells was also confirmed by the fluorescence analysis of the OM fraction,20 where less flavin contents were observed for the OM fraction of $\Delta omcBEST$ cells compared with that of WT (Fig. S11[†]). However, in the system without *omcBEST* genes, the RF peak current at -0.125 V and I_c still showed a positive correlation with a comparable slope value to WT (Fig. S9c[†]). This result suggests that, in addition to omcBEST proteins, other types of OM c-Cyts in the Geobacter WT strain associate with RF to mediate EET (Fig. 4). This situation differs from the EET process reported for S. oneidensis MR-1, which is unable to bind FMN in the absence of a single binding protein (MtrC).14 This finding is also consistent with the wider variety of c-Cyts encoded in the genome of G. sulfurreducens compared to that of S. oneidensis MR-1.²¹ Together, these results confirm that RF and FMN associate with OM c-Cyts of G. sulfurreducens and serve as redox cofactors (Fig. 4), and that Geobacter is more flexible with respect to flavin uptake and binding mechanisms than S. oneidensis MR-1. In addition, because the peak current for bound-RF decreased when I_c was reduced by the addition of a metabolic inhibitor (Fig. 2d), reduced hemes in OM c-Cyts of G. sulfurreducens play an important role in the interaction with flavins, as has also been reported in MR-1.15

Conclusions

We have provided the first experimental evidence for the involvement of self-secreted flavin in EET in G. sulfurreducens. As the flavin uptake mechanism in G. sulfurreducens appears to be similar to that of S. oneidensis MR-1,14 and that a wide range of microorganisms possesses homologous OM c-Cyt protein complexes to these two species,^{2,22} flavin may be a universal factor for efficient EET at bacteria/solid interfaces. In addition, if reduced hemes in OM c-Cyts govern the interaction with flavin, the intracellular redox homeostasis of Geobacter cells could be maintained by the release and binding of flavin, as is observed in MR-1,15 given that the oxidation state of hemes in OM *c*-Cyts reflects the balance between the electron input from respiration and the output by EET. Our present experiments were focused on monolayers of cells, where conductive filaments (nanowires) were not present; thus, it will be of great interest to examine the interaction of flavins with conductive filaments in thick biofilms, where these filaments may also play a role in intercellular EET. Recent theoretical analysis of charge flow along conductive filaments highlighted the importance of a multi-step hopping transport mechanism with charge localizing sites separated by less than 1 nm and reorganization energies lower than those known in biology.13 As arrays of OM c-Cyts such as OmcS expressed on the conductive filaments of G. sul*furreducens* have been implicated as charge carriers,²³ a specific flavin association with c-Cyts could also play an extensive role in multi-step charge hopping through thick biofilms. Further studies on the uptake of self-secreted flavins as the boundcofactor of OM c-Cyts will serve for the mechanistic understanding of the complex EET reactions and also for optimization of microbial fuel cells6 and the bioremediation of waste waters and contaminated sediments.7

Acknowledgements

We thank Prof. D. R. Lovley of the University of Massachusetts for kindly providing the $\Delta omcBEST$ mutant. This work was financially supported by a Grant-in-Aid for Specially Promoted Research of the Japan Society for Promotion of Science (JSPS) KAKENHI Grant Number 24000010.

References

- K. H. Nealson and D. Saffarini, Annu. Rev. Microbiol., 1994, 48, 311; D. R. Lovley, Annu. Rev. Microbiol., 2012, 66, 391.
- J. K. Fredrickson, M. F. Romine, A. S. Beliaev, J. M. Auchtung, M. E. Driscoll, T. S. Gardner, K. H. Nealson, A. L. Osterman, G. Pinchuk, J. L. Reed, D. A. Rodionov, J. L. M. Rodrigues, D. A. Saffarini, M. H. Serres, A. M. Spormann, I. B. Zhulin and J. M. Tiedje, *Nat. Rev. Microbiol.*, 2008, 6, 592.
- 3 S. Matsuda, H. Liu, S. Kato, K. Hashimoto and S. Nakanishi, *Environ. Sci. Technol.*, 2011, 45, 10163; H. W. Harris, M. Y. El-Naggar, O. Bretschger, M. J. Ward, M. F. Romine, A. Y. Obraztsova and K. H. Nealson, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, 107, 326.

- 4 Z. M. Summers, H. E. Fogarty, C. Leang, A. E. Franks, N. S. Malvankar and D. R. Lovley, *Science*, 2010, **330**, 1413; R. Nakamura, F. Kai, A. Okamoto, G. J. Newton and K. Hashimoto, *Angew. Chem., Int. Ed.*, 2009, **48**, 508.
- 5 S. Kato, K. Hashimoto and K. Watanabe, *Proc. Natl. Acad. Sci.* U. S. A., 2012, **109**, 10042.
- 6 D. R. Lovley, *Nat. Rev. Microbiol.*, 2006, 4, 497; U. Schroder,
 J. Niessen and F. Scholz, *Angew. Chem., Int. Ed.*, 2003, 42, 2880.
- 7 B. K. Amos, Y. Sung, K. E. Fletcher, T. J. Gentry, W. M. Wu,
 C. S. Criddle, J. Zhou and F. E. Loffler, *Appl. Environ. Microbiol.*, 2007, 73, 6898.
- 8 H. T. Dinh, J. Kuever, M. Mussmann, A. W. Hassel, M. Stratmann and F. Widdel, *Nature*, 2004, **427**, 829.
- 9 D. K. Newman and R. Kolter, Nature, 2000, 405, 94.
- 10 E. Marsili, D. B. Baron, I. D. Shikhare, D. Coursolle, J. A. Gralnick and D. R. Bond, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 3968.
- T. A. Clarke, M. J. Edwards, A. J. Gates, A. Hall, G. F. White, J. Bradley, C. L. Reardon, L. Shi, A. S. Beliaev, M. J. Marshall, Z. M. Wang, N. J. Watmough, J. K. Fredrickson, J. M. Zachara, J. N. Butt and D. J. Richardson, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 9384; D. J. Richardson, J. N. Butt and T. A. Clarke, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 7537; H. Liu, G. J. Newton, R. Nakamura, K. Hashimoto and S. Nakanishi, *Angew. Chem., Int. Ed.*, 2010, **49**, 6596.
- N. S. Malvankar, M. Vargas, K. P. Nevin, A. E. Franks, C. Leang, B.-C. Kim, K. Inoue, T. Mester, S. F. Covalla, J. P. Johnson, V. M. Rotello, M. T. Tuominen and D. R. Lovley, *Nat. Nanotechnol.*, 2011, 6, 573; Y. A. Gorby, S. Yanina, J. S. McLean, K. M. Rosso, D. Moyles, A. Dohnalkova, T. J. Beveridge, I. S. Chang, B. H. Kim, K. S. Kim, D. E. Culley, S. B. Reed, M. F. Romine, D. A. Saffarini, E. A. Hill, L. Shi, D. A. Elias, D. W. Kennedy, G. Pinchuk, K. Watanabe, S. Ishii, B. Logan, K. H. Nealson and J. K. Fredrickson, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, 103, 11358.
- 13 N. F. Polizzi, S. S. Skourtis and D. N. Beratan, *Faraday Discuss.*, 2012, 155, 43.
- 14 D. R. Bond and D. R. Lovley, *Appl. Environ. Microbiol.*, 2003, 69, 1548.
- 15 A. Okamoto, K. Hashimoto, K. H. Nealson and R. Nakamura, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 7856.
- 16 N. J. Kotloski and J. A. Gralnick, mBio, 2013, 4, 1.
- 17 H. von Canstein, J. Ogawa, S. Shimizu and J. R. Lloyd, *Appl. Environ. Microbiol.*, 2008, **74**, 615.
- 18 G. Reguera, K. P. Nevin, J. S. Nicoll, S. F. Covalla, T. L. Woodard and D. R. Lovley, *Appl. Environ. Microbiol.*, 2006, 72, 7345.
- K. Inoue, C. Leang, A. E. Franks, T. L. Woodard, K. P. Nevin and D. R. Lovley, *Environ. Microbiol. Rep.*, 2011, 3, 211;
 J. W. Voordeckers, B. C. Kim, M. Izallalen and D. R. Lovley, *Appl. Environ. Microbiol.*, 2010, 76, 2371; C. Leang, X. Qian, T. Mester and D. R. Lovley, *Appl. Environ. Microbiol.*, 2010, 76, 4080.

- 20 K. Inoue, X. L. Qian, L. Morgado, B. C. Kim, T. Mester, M. Izallalen, C. A. Salgueiro and D. R. Lovley, *Appl. Environ. Microbiol.*, 2010, 76, 3999.
- B. A. Methe, K. E. Nelson, J. A. Eisen, I. T. Paulsen, W. Nelson, J. F. Heidelberg, D. Wu, M. Wu, N. Ward, M. J. Beanan, R. J. Dodson, R. Madupu, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, M. Gwinn, J. F. Kolonay, S. A. Sullivan, D. H. Haft, J. Selengut, T. M. Davidsen, N. Zafar, O. White, B. Tran, C. Romero, H. A. Forberger, J. Weidman, H. Khouri, T. V. Feldblyum, T. R. Utterback, S. E. Van Aken, D. R. Lovley and C. M. Fraser, *Science*, 2003, **302**, 1967; J. F. Heidelberg, I. T. Paulsen, K. E. Nelson, E. J. Gaidos, W. C. Nelson, T. D. Read, J. A. Eisen,
- R. Seshadri, N. Ward, B. Methe, R. A. Clayton, T. Meyer, A. Tsapin, J. Scott, M. Beanan, L. Brinkac, S. Daugherty, R. T. DeBoy, R. J. Dodson, A. S. Durkin, D. H. Haft, J. F. Kolonay, R. Madupu, J. D. Peterson, L. A. Umayam, O. White, A. M. Wolf, J. Vamathevan, J. Weidman, M. Impraim, K. Lee, K. Berry, C. Lee, J. Mueller, H. Khouri, J. Gill, T. R. Utterback, L. A. McDonald, T. V. Feldblyum, H. O. Smith, J. C. Venter, K. H. Nealson and C. M. Fraser, *Nat. Biotechnol.*, 2002, **20**, 1118.
- 22 J. E. Butler, N. D. Young and D. R. Lovley, *BMC Genomics*, 2009, **10**, 103.
- 23 C. Leang, X. L. Qian, T. Mester and D. R. Lovley, *Appl. Environ. Microbiol.*, 2010, **76**, 4080.