Electron Communication of *Bacillus subtilis* in Harsh Environments

**HIGHLIGHTS**

- *B. subtilis* maintains electrochemical activity at pH = 1.5 for over 2 years
- *B. subtilis* maintains electrochemical activity up to 100°C for hours
- NAD acts as an essential participant in electron communication of *B. subtilis*
SUMMARY
Elucidating the effect of harsh environments on the activities of microorganisms is important in revealing how microbes withstand unfavorable conditions or evolve mechanisms to counteract those effects, many of which involve electron transfer phenomena. Here we show that the non-acidophilic and non-thermophilic Bacillus subtilis is able to maintain activity after being subjected to extreme temperatures (100 °C for up to 8 h) and acidic environments (pH = 1.50 for over 2 years). In the process, our results suggest that B. subtilis utilizes an extracellular electron transfer as an electron communication pathway between B. subtilis and the environment that involves the cofactor nicotinamide adenine dinucleotide as an essential participant to maintain viability. Elucidation of the capability of the non-acidophilic and non-thermophilic strain to maintain viability under these extreme conditions could aid in understanding the cell responses to different environments from the perspective of energy conservation pathways.

INTRODUCTION
Whether a microorganism is autotrophic or heterotrophic, free living or obligate parasite, energy generation is essential for the cell to survive (Hernandez and Newman, 2001). Energy metabolism is dominated by oxidation-reduction reactions, in which electron transfer plays a fundamental role, supplying the reducing power and maintaining the intracellular redox balance through the regeneration of the redox cofactor nicotinamide adenine dinucleotide (NAD) (Li et al., 2018; Nealson and Rowe, 2016). Extracellular electron transfer (EET) is the process by which electrons generated by microbial metabolism are transported to extracellular substrates that act as electron acceptors. Different EET mechanisms have been identified, including direct electron transfer via redox proteins, such as membrane-bound c-type cytochromes (Pirbadian et al., 2014; Reguera et al., 2005), or indirect electron transfer via secreted redox molecules, such as flavins (Marsili et al., 2008), phenazines (Wang et al., 2010), and quinones (Sasaki et al., 2014).

Harsh environments will, in general, denature proteins and suppress microbial activity, and even subtle changes in the structure of sensitive proteins may result in loss of the cell’s ability to communicate with the environment. Bacteria are often subjected to various environmental stresses, among which the most important variables are temperature and pH. Both strongly affect bacterial metabolism and electron transfer (Courbet et al., 2010), but the latter has not been studied in detail for bacteria at high temperatures and low pH conditions. Elucidation of the mechanisms involved in EET has been the subject of widespread attention, as it is essential in the understanding of natural processes such as biogeochemical cycling as well as in the development and optimization of many applications, ranging from biofuel production to bioremediation systems (Chen et al., 2012; Collier and Mrksich, 2006; Lan et al., 2018; Marsili et al., 2008; Nielsen et al., 2010; Pfeffer et al., 2012; Wang et al., 2018; Wu et al., 2018). Hence, it is imperative to understand how microorganisms manage to retain electron transfer capabilities between intracellular and extracellular environments under extreme conditions.

B. subtilis is an aerobic, gram-positive bacterium, with an ample metabolic repertoire, is widely present in soil and aquatic environments, and is a key player in environmental processes and in applications in processes of medical and biotechnological interest (Beauregard et al., 2013). B. subtilis provides an accessible model for investigating the response of electron transfer of gram-positive bacteria to environmental stress. Sporulation initiates in response to harsh environment through a six-stage process that lasts approximately 8 h. Even though spores have very low metabolic activity (Church and Halvorson, 1957; Ghosh et al., 2015; Segev et al., 2012), they still have to possess the ability to communicate with the environment to initiate germination and become a vegetative cell. So we conjecture that EET is a pathway used by B. subtilis to...
communicate with the environment as this knowledge will also contribute to elucidate the EET pathway in gram-positives in general. Despite the large corpus of metabolic and physiological studies, there is limited information on energy metabolism and EET when non-acidophilic and non-thermophilic \textit{B. subtilis} is subjected to harsh conditions.

In this work, we analyzed the electrochemical activity of \textit{B. subtilis} under two conditions: low pH and high temperatures. In a series of controlled experiments, we assessed the electrochemical activity of \textit{B. subtilis} kept at \( \text{pH} = 1.50 \) over 2 years, whereas in another series of experiments we evaluated the electrochemical activity of \textit{B. subtilis} in suspensions incubated at 100°C for various periods. Cyclic voltammetry (CV), differential pulse voltammetry (DPV), and chronoamperometry (CA) were used to determine the electrochemical activity and the EET ability of \textit{B. subtilis}, whereas ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS) was employed to identify low-molecular-weight redox molecules. Our results show that the electrogenic activity of \textit{B. subtilis} remains after long-term exposure to harsh environmental conditions, and they also demonstrate the role of NAD in the EET mechanism.

**RESULTS**

**Redox Activity after High-Temperature Treatment**

To investigate the effect of high temperature on electron transfer between the microbial cells and electrodes, a series of tests were performed. \textit{B. subtilis} was incubated at 26°C and 100°C, and its electrochemical activity was assessed by CV (Figure 1). Bare glassy carbon electrode showed no redox peaks (Figure 1, curve a), whereas oxidation peaks at +0.39 V and +0.09 V and reduction peaks at +0.26 V and −0.06 V were observed for \textit{B. subtilis} incubated at 26°C (Figure 1, curve b). Interestingly, significant redox currents were produced by \textit{B. subtilis} after treatment at 100°C for 3 h (Figure 1, curve c), but the redox peaks at −0.06 V and +0.09 V disappeared after 8 h at 100°C (Figure 2A). These observations suggest that the mechanism responsible for the changes in current with temperature involves redox compounds or proteins that retain electrochemical activity after being subjected to high temperatures (Figure 2B).

Although temperature has a significant effect on bacterial physiology and metabolism, other environmental factors, such as pH, severely affect bacterial functions. To further elucidate the effect of harsh environments on electron transfer, we studied the redox activity of \textit{B. subtilis} at various pH values also. Experiments were carried out over a range of pH values in the range 1.50–12.00. When \textit{B. subtilis} is grown in the medium used in the experiments, the pH of the culture reaches a stable value of 4.68, so we first use this condition for the electrochemical tests. At \( \text{pH} = 4.68 \), redox peaks were observed at +0.39 V and +0.26 V and at +0.09 V and −0.06 V, whereas at \( \text{pH} = 1.50 \) these redox peaks showed a positive shift (Figure 3). The shift in the negative direction upon an increase in pH value is consistent with the involvement of protons in the reaction studied, as indicated by the Nernst equation (Table S2). The electrochemical activity of the redox peaks at +0.39 V and +0.26 V remained stable within the pH range 1.50–10.60, but no redox peaks were observed at \( \text{pH} = 12.00 \) (Figure S5). These results suggest that extreme alkaline conditions may cause inhibition of the redox substance. However, this inhibition seems to be reversible; when the electrode was placed again in \( \text{pH} = 4.68 \) buffer after electrochemical testing at \( \text{pH} 12.00 \), all the redox peaks reappeared at the same positions as in earlier experiments. This feature may play a role in the mechanisms that ensure the survival of \textit{B. subtilis} subjected to extreme conditions.
To investigate the long-term stability in acidic conditions, *B. subtilis* was suspended in pH = 1.50 buffer for 736 days. After this prolonged treatment, the low-pH redox peak currents at +0.57 V and +0.41 V and at +0.12 V and +0.06 V were still present (Figures 3 and S6). These data showed that *B. subtilis* redox ability remains stable even after very long-term exposure to low-pH conditions.

Our data show that *B. subtilis* is able to maintain the redox ability after treatment at high temperature and for long term in low pH, but it cannot directly demonstrate the presence of EET, i.e., the redox reaction of DPV or CV does not always mean the active state of live bacteria. To this end, CA was used to evaluate the effect of acetate as a substrate on *B. subtilis*, and because only viable bacteria or spores can respond to acetate, the data will confirm live *B. subtilis* contributing to current change.

Based on the CV data, potentials at +0.15 V and +0.50 V were chosen to perform CA analysis. When the potential was held at +0.15 V, the current due to the oxidation reaction at +0.09 V changed when acetate was added to the system, i.e., we held +0.15 V to determine if the electrons from acetate degradation are able to transport at +0.09 V (not at +0.39 V). In control experiments with a bare glassy carbon electrode, the stable current is 0.50 nA at 1800 s. Upon addition of acetate to the system, an increase in current of 0.10 nA was observed, caused by the perturbation of the electrolyte. In the experiments with the *B. subtilis* electrode, the stable current was 1.50 nA, which increased to 3.50 nA after addition of acetate (Figure 4A). This increase could be explained by changes in the bacterial capacitance, suggesting that the oxidation peak at +0.09 V has a limited contribution to the EET pathway.

When the potential is held at +0.50 V, a current change may be caused by the two oxidation reactions, i.e., at +0.09 V and +0.39 V, or only one of them. As a control, a stable current of 2.90 nA was observed at 2,120 s in the glassy carbon held at +0.50 V, which increased by ca. 0.50 nA after addition of acetate. However, the
current measured in the B. subtilis electrode was 24 nA, increasing to 39 nA upon addition of acetate (Figure 4B). In the B. subtilis electrode system, the current measured at +0.50 V is therefore 10 times higher than that at +0.15 V. Compared with the data with B. subtilis at +0.15 V, the redox reaction at +0.39 V plays a major role in the observed current increase, strongly suggesting that the EET pathway in B. subtilis involves the oxidation peak at +0.39 V. Hence the redox substance involved at +0.39 V needs to be identified.

Lysozyme test was used for verifying whether the redox peaks come from vegetative cells, spores, or both. Spores are resistant to lysozyme, whereas vegetative cells are disrupted by the enzyme. Lysozyme does not show any redox activity in this condition as shown in the Figure 5, curve a. After lysozyme treatment the remnant of cell debris or contents of cellular materials enter into the supernatant, and their electrochemistry signal originates from B. subtilis. In Figure 5, curve b, only the redox peaks at −0.06 and +0.09 V are observed in the lysozyme-treated supernatant, suggesting that the peaks at −0.06 V and +0.09 V may be due to the redox activity of the spores, whereas the disappearance of the peaks at +0.39 V and +0.26 V indicates that these originate by the redox activity of intact vegetative cells.

The results in Figure 6 show that both supernatant and pellet present the same redox peaks after treatment. All the redox peaks were observed in the supernatant after a high-temperature or acid treatment (Figure S7), suggesting that a molecule with redox activity is secreted to the extracellular medium. To avoid interferences, the identification of redox substances was performed only in supernatants. After exposure to low pH for 736 days, the electrochemical response shown by B. subtilis implies the existence of an electron transport that permits the adaptation of the microorganism to harsh environments.

To identify redox substances that might be involved in EET, we analyzed the composition of the supernatant by UPLC-Q-TOF MS. The fragment ion at m/z 663.4566 corresponds to NAD⁺, whereas the fragment ion at m/z 685.4349 corresponds to NAD⁺-H + Na. In Figure 7B NAD⁺ ion intensity is slightly lower than that in Figure 7C, compatible with NAD⁺ being a heat-stable molecule (Chini et al., 2017) with higher stability in acidic conditions than at pH = 7.00 (Gorton and Domínguez, 2007). The fragment ions at m/z 664.4574 and 665.4590 correspond to NAD⁺ isotopic peak NAD⁺+H and NAD⁺+2H, respectively; The fragment ions 686.4415 and 687.4434 correspond to NAD⁺-H + Na isotopic peaks NAD⁺+Na and NAD⁺+Na + H, respectively. These results are consistent with the mass spectrum observed using NAD⁺ as standard (Figure S8).

To confirm the participation of NAD⁺ in the electrochemical activity of B. subtilis, we performed DPV tests in supernatants spiked with NAD⁺ standard (Figures 8 and S9). The supernatant showed an obvious oxidation peak at +0.30 V. After addition of 20 μM NAD⁺ at pH = 4.68, the current at the peak increased. The combined data from MS and DPV strongly suggest that NAD⁺ is indeed involved in EET.

Our results show that the gram-positive B. subtilis is able to maintain electron transfer activity after being subjected to extreme temperatures and acidic environments for very long times. The results observed after
exposure at low pH suggest that the bacteria are able to keep their electron communication pathways active, adapting to the acid environment to maintain their viability. Our analysis suggests that the redox peaks at +0.39 V and +0.26 V can be assigned to NAD+/NADH, and that the EET in B. subtilis involves NAD as an essential participant in the process.

**DISCUSSION**

**High-Temperature Effect**

Most *Bacillus* species present a certain degree of thermostability and are able to respond to environmental stresses by triggering metabolic and physiological changes. One of these involves the release of extracellular polymeric substances (EPS). EPS can be proteins, nucleic acids, lipids, or other biopolymers, with some of the proteins presenting enzymatic activities and redox functions (Bengtsson et al., 1999; Los and Murata, 2004; Morokutti et al., 2005; Rothschild and Mancinelli, 2001; Xiao et al., 2017). The redox peaks observed at −0.06 V and +0.09 V in Figure 1 (curve b) may be attributed to membrane-bound redox proteins, as we have previously reported (Xiao et al., 2017). Raman spectroscopy revealed cytochrome c at the surface of bacteria (Table S1). The addition of EDTA, a known Fe³⁺ chelator, caused the inactivation of cytochrome c (Figure S1). Finally, SDS-PAGE analysis shows a band of molecular weight approximately 38 kDa (Figure S2), which may correspond to subunit II of cytochrome c (Bengtsson et al., 1999). These results are consistent with the structural features of cytochrome c.

![Figure 4. Current Responses of B. subtilis to Addition of 30 mM Acetate in pH 4.68 Phosphate Buffer](image-url)

(A) The potential for CA measurements (not stirred) is +0.15 V versus Ag/AgCl.
(B) The potential for CA measurements (not stirred) is +0.50 V versus Ag/AgCl. The arrow indicates addition of 200 μL sodium acetate solution (inset: current response of a bare electrode).
Heating can enhance molecular movements, which may accelerate EPS disaggregation or dissolution (Figure S4). This could facilitate direct contact between membrane-bound cytochrome $c$ and the electrode, thus enabling electron transfer. However, increasing exposure time at 100°C leads to decreased peak currents, which then disappeared at long-term exposure. This may be explained by the fluidization of membranes caused by high temperatures and the consequent disintegration of the lipid bilayer (Los and Murata, 2004; Rothschil d and Mancinelli, 2001), resulting in the decline of the redox activity of cytochrome $c$.

Low-pH Treatment
Under stress conditions, $B.\ subtilis$ is able to initiate many survival mechanisms such as motility, uptake of exogenous DNA, and sporulation (Tan and Ramamurthi, 2014). When Bacillus is under extreme nutrient deprivation, it differentiates into spores, extremely resistant to potentially damaging environmental conditions (Barney and Austin, 2017). In the case of low-pH stress, to maintain metabolic activity, bacteria need to keep pH homeostasis. This mechanism requires more energy than in neutral pH environment, provided by the activity of the electron transport chain (Lund et al., 2014). Low pH can trigger spore germination and initiation of vegetative growth in $B.\ subtilis$ (Wilks et al., 2009) and is linked to an increase in NADH oxidase activity in germinated spores and in the upregulation of NAD(P)-dependent dehydrogenases. These phenomena accelerate electron transfer and the pumping of protons out of the cell to maintain internal pH homeostasis (Wilks et al., 2009).

Electron Communication Pathway
In an attempt to elucidate the electron transport mechanism, we performed experiments to identify the redox molecule(s) involved in EET. $B.\ subtilis$ can secrete flavins (Morokutti et al., 2005), which have been reported as electron transfer mediators for other bacteria, e.g., Shewanella. However, in this study, flavins may not appear to make a significant contribution to electron transfer because the culture medium contains $Cu^{2+}$, which is known to form a stable complex with flavin through d-$\pi$ back donation and may

---

**Figure 5. Cyclic Voltammograms of Lysozyme Treated $B.\ subtilis$**

Cyclic voltammograms of lysozyme at bare glassy carbon electrode (curve a) and lysozyme-treated $B.\ subtilis$ supernatant (curve b).

**Figure 6. Cyclic Voltammograms of $B.\ subtilis$ Pellets and Supernatant**

Cyclic voltammograms of pellets and supernatant. $B.\ subtilis$ were centrifuged at 5,000 g for 10 min to obtain the pellets and then washed three times, and the resuspended pellets were then incubated for 5 h at 26°C. pellets (black), supernatant (red). Identifying redox substances of $B.\ subtilis$. 

---
make inactive the redox reaction of flavin. When CuSO₄ and guaiacol are added to lysogeny broth medium, flavin was not detected in our experiments.

Most energy-producing processes require coenzymes such as NAD, a highly abundant cellular component of bacteria that participates in electron transfer during oxidation-reduction reactions, which convert the oxidized form NAD into NADH, a strong reducing agent (Chini et al., 2017; Kido et al., 2015). NAD is
oxidized or reduced by the loss or gain of two electrons, in reactions involving the removal of two hydrogen atoms (a "hydride ion" and a proton). NAD also regulates numerous NAD+/NADH-dependent enzymes, including dehydrogenases. NADH and NAD+ can be transported across cell plasma membranes, and it has been suggested that extracellular NAD+ may act as a signaling molecule (Ying, 2006). NAD+ is a heat-stable molecule (Chini et al., 2017), presenting higher stability in acidic conditions than at pH = 7.00 (Gorton and Domínguez, 2007), The nicotinamide moiety in NAD presents a planar structure, whereas it is puckered in NADH (Fjeld et al., 2003). The difference in structure leads to inhibition of the redox function at extreme alkaline conditions, which is consistent with the results observed in the CV at different pH values (Figure S5).

The MS results shown above suggest that NAD+ was secreted into the solution. Temperature not only influences bacterial growth rate, enzyme activity, cell composition, and nutritional requirements but also has effects on the solubility of solute molecules, ion transport and diffusion, osmotic effects on membranes, surface tension, and electron transfer (Beales, 2004). NAD is present in Bacillus vegetative cell and spores (Setlow and Setlow, 1977). B. subtilis spores show resistance to high temperature, and NAD+ is a heat-stable molecule (Chini et al., 2017). NAD, a key class of cofactors, serves as essential electron donor or acceptor in all biological organisms (Liu et al., 2018) and drives major catabolic and anabolic reactions to maintain cellular redox homeostasis and energy metabolism (Xiao et al., 2018). NAD(H/+) is a considerable source of the intracellular electron pool from which intracellular electrons are transferred to extracellular electron acceptors via EET pathways (Li et al., 2018).

Intact bacteria can release NADH into the extracellular medium, and up to 70% of NADH release occurs during the exponential growth phase (Wos and Pollard, 2009). Secretion of NAD to the extracellular milieu
has been reported in diverse species, such as *E. coli*, *Rhodobacter capsulatus* (Ying, 2006), and microorganisms in activated sludge (Wos and Pollard, 2009), as well as by wood-degrading fungi and animal cells (Kido et al., 2015; Xiao et al., 2018). The redox activity of this molecule suggests its participation in efficient electron transport and extracellular turnover of NADH to NAD\(^+\) (Wos and Pollard, 2009).

The study of EET is essential owing to its importance in biological fuel cells, biogeochemical cycling, and bioremediation processes. Elucidation of the capability of *B. subtilis* to maintain viability under extreme conditions could help in understanding bacterial responses to different environments from the perspective of energy conservation and electron transfer.

**Limitations of the Study**
In harsh environments, we studied the electron communication between *B. subtilis* and environment and finally identified NAD as an essential participant in this process. Further work is needed to confirm if cytochrome c plays a key role in direct electron transfer. Moreover, flavins may appear to make a contribution to electron transfer when the culture medium of *B. subtilis* does not contain Cu\(^{2+}\).

**METHODS**
All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Transparent Methods, nine figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.isci.2019.01.020.

**ACKNOWLEDGMENTS**
This study is financially supported by National Natural Science Foundation of China (21777155) and Fujian STS Project (2016T3032). C.A.-R. was partially supported by grant BB/J01916X from the UK’s Biotechnology and Biological Sciences Research Council (BBSRC).

**AUTHOR CONTRIBUTIONS**
F.Z. and L.C. designed research and wrote the manuscript; L.C. performed cultivation, and C.C. performed electrochemistry analyses. S.W. performed proteome analyses. J.R.V., R.C.T.S, and C.A.-R. revised the manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

Received: September 5, 2018  
Revised: January 11, 2019  
Accepted: January 16, 2019  
Published: February 22, 2019

**REFERENCES**
Barney, B.L., and Austin, D.E. (2017). Dynamics of rebounding *Bacillus subtilis* spores determined using image-charge detection. J. Biol. Phys. 43, 481–492.

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. Compr. Rev. Food Sci. Food Saf. 3, 1–20.

Beauregard, P.B., Chai, Y., Vlamakis, H., Losick, R., and Kolter, R. (2013). *Bacillus subtilis* biofilm induction by plant polysaccharides. Proc. Natl. Acad. Sci. U S A 110, E1621–E1630.

Bengtsson, J., Talisma, H., Rivolta, C., and Hednerstedt, L. (1999). Subunit II of *Bacillus subtilis* cytochrome c oxidase is a lipoprotein. J. Bacteriol. 181, 685–688.

Chen, L.X., Xiao, Y., and Zhao, F. (2012). Biocathodes in microbial fuel cells, Prog. Chem. 24, 157–162.

Chini, C.C.S., Tarragé, M.G., and Chini, E.N. (2017). NAD and the aging process: role in life, death and everything in between. Mol. Cell Endocrinol. 455, 62–74.

Church, B.D., and Halvorson, H. (1957). Intermediate metabolism of aerobic spores. I. Activation of glucose oxidation in spores of *Bacillus cereus* var. terminalis. J. Bacteriol. 73, 470–476.

Collier, J.H., and Mrksich, M. (2006). Engineering a biospecific communication pathway between cells and electrodes. Proc. Natl. Acad. Sci. U S A 103, 2021–2025.

Cournet, A., Délia, M.L., Bérge, A., Roques, C., and Bérge, M. (2010). Electrochemical reduction of oxygen catalyzed by a wide range of bacteria including Gram-positive. Electrochem. Commun. 12, 505–508.

Field, C.C., Birdsong, W.T., and Goodman, R.H. (2003). Differential binding of NAD\(^+\) and NADH allows the transcriptional corepressor carboxyl-terminal binding protein to serve as a metabolic sensor. Proc. Natl. Acad. Sci. U S A 100, 9202–9207.
Ghosh, S., Korza, G., Maciejewski, M., and Setlow, P. (2015). Analysis of metabolism in dormant spores of bacillus species by 31P Nuclear magnetic resonance analysis of low-molecular-weight compounds. J. Bacteriol. 197, 992–1001.

Gorton, L., and Dominguez, E. (2007). Electrochemistry of NAD(P)+/NAD(P)H. In Encyclopedia of Electrochemistry, A.J. Bard, ed., pp. 67–143, https://doi.org/10.1002/9785327610626.bard00004.

Hernandez, M.E., and Newman, D.K. (2001). Extracellular electron transfer. Cell. Mol. Life Sci. 58, 1562–1571.

Kido, R., Takeeda, M., Manabe, M., Miyagawa, Y., Itakura, S., and Tanaka, H. (2015). Presence of extracellular NAD+ and NADH in cultures of wood-degrading fungi. Biocontrol Sci. 20, 105–113.

Lan, T.-H., Wang, C.-T., Sangeetha, T., Yang, Y.-C., and Garg, A. (2018). Constructed mathematical model for nanowire electron transfer in microbial fuel cells. J. Power Sources 402, 483–488.

Li, F., Li, Y.-X., Cao, Y.-X., Wang, L., Liu, C.-G., Shi, L., and Song, H. (2018). Modular engineering to increase intracellular NAD(H)+ promotes rate of extracellular electron transfer of Shewanella oneidensis. Nat. Commun. 9, 3637.

Liu, J., Li, H., Zhao, G., Caiyin, Q., and Qiao, J. (2018). Redox cofactor engineering in industrial microorganisms: strategies, recent applications and future directions. J. Ind. Microbiol. Biotechnol. 45, 313–327.

Los, D.A., and Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. Biochim. Biophys. Acta 1666, 142–157.

Lund, P., Tramonti, A., and De Biase, D. (2014). Coping with low pH: molecular strategies in neutralophilic bacteria. FEMS Microbiol. Rev. 38, 1091–1125.

Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., and Bong, D.R. (2008). Shewanella secretes flavins that mediate extracellular electron transfer. Proc. Natl. Acad. Sci. U S A 105, 3968–3973.

Morokutti, A., Lyskowski, A., Sollner, S., Pointner, E., Fitzpatrick, T.B., Krakly, C., Gruber, K., and Muchovej, P. (2005). Structure and function of YcnD from Bacillus subtilis, a flavin-containing oxidoreductase. Biochemistry 44, 13724–13733.

Nealson, K.H., and Rowe, A.R. (2016). Electromicrobiology: realities, grand challenges, goals and predictions. Microb. Biotechnol. 9, 595–600.

Nielsen, L.P., Riisgaard-Petersen, N., Fossing, H., Christensen, P.B., and Sayama, M. (2010). Electric currents couple spatially separated biogeochemical processes in marine sediment. Nature 463, 1071–1074.

Pfeffer, C., Larsen, S., Song, J., Dong, M., Besenbacher, F., Meyer, R.L., Kjeldsen, K.U., Schreiber, L., Gorby, Y.A., and El-Naggar, M.Y. (2012). Filamentous bacteria transport electrons over centimetre distances. Nature 497, 218–221.

Pirbadian, S., Barchinger, S.E., Leung, K.M., Byun, H.S., Jangir, Y., Bouhenni, R.A., Reed, S.B., Romine, M.F., Saffarini, D.A., Shi, L., et al. (2014). Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. Proc. Natl. Acad. Sci. U S A 111, 12883–12888.

Requena, G., McCarthy, K.D., Mehta, T., Ncill, J.S., Tuominen, M.T., and Lovley, D.R. (2005). Extracellular electron transfer via microbial nanowires. Nature 435, 1098–1101.

Rothschild, L.J., and Mancinelli, R.L. (2001). Life in extreme environments. Nature 409, 1092–1101.

Sasaki, K., Tsuge, Y., Sasaki, D., and Kondo, A. (2014). Increase in lactate yield by growing Corynebacterium glutamicum in a bioelectrochemical reactor. J. Biosci. Bioeng. 117, 598–601.

Segev, E., Smith, Y., and Ben-Yehuda, S. (2012). RNA dynamics in aging bacterial spores. Cell 148, 139–149.

Setlow, B., and Setlow, P. (1977). Levels of oxidized and reduced pyridine nucleotides in dormant spores and during growth, sporulation, and spore germination of Bacillus megaterium. J. Bacteriol. 129, 857–865.

Tan, I.S., and Ramamurthi, K.S. (2014). Spore formation in Bacillus subtilis. Environ. Microbiol. Rep. 6, 212–225.

Wang, C.-T., Sangeetha, T., Zhao, F., Garg, A., Chang, C.-T., and Wang, C.-H. (2018). Sludge selection on the performance of sediment microbial fuel cells. Int. J. Energ. Res. 42, 4250–4255.

Wang, Y., Kern, S.E., and Newman, D.K. (2010). Endogenous phenazone antibiotics promote anaerobic survival of Pseudomonas aeruginosa via extracellular electron transfer. J. Bacteriol. 192, 365–369.

Wilks, J.C., Kitko, R.D., Cleeton, S.H., Lee, G.E., Ugwu, C.S., Jones, B.D., BonDurant, S.S., and Sionczewski, J.L. (2009). Acid and base stress and transcriptomic responses in Bacillus subtilis. Appl. Environ. Microbiol. 75, 981–990.

Wos, M., and Pollard, P. (2009). Cellular nicotinamide adenine dinucleotide (NADH) as an indicator of bacterial metabolic activity dynamics in activated sludge. Water Sci. Technol. 60, 783–791.

Wu, J.C., Yan, W.-M., Wang, C.-T., Wang, C.-H., Pai, Y.-H., Wang, K.-C., Chen, Y.-M., Lan, T.-H., and Thangavel, S. (2018). Treatment of oily wastewater by the optimization of fe2o3 calcination temperatures in innovative bio-electron-fenton microbial fuel cells. Energies 11, 565.

Xiao, W., Wang, R.S., Handy, D.E., and Loscalzo, J. (2018). NAD(H) and NADPH redox couples and cellular energy metabolism. Antioxid. Redox Signal. 28, 251–272.

Xiao, Y., Zhang, E., Zhang, J., Dai, Y., Yang, Z., Christensen, H.E.M., Ulstrup, J., and Zhao, F. (2017). Extracellular polymeric substances are transient media for microbial extracellular electron transfer. Sci. Adv. 3, e1700623.

Yang, W. (2006). NAD+ and NADH in cellular functions and cell death. Front. Biosci. 71, 3129–3148.
Supplemental Information

Electron Communication of *Bacillus subtilis* in Harsh Environments

Lixiang Chen, Changli Cao, Shuhua Wang, John R. Varcoe, Robert C.T. Slade, Claudio Avignone-Rossa, and Feng Zhao
Supplemental Information

Transparent method

Figure S1. Cyclic voltammogram of *B. Subtilis* treated with EDTA to chelate Fe$^{3+}$ to inactivate cytochrome c and inhibit activity

Figure S2. SDS-PAGE analysis of *B. subtilis* protein

Figure S3. Raman spectra of pellet

Table S1. Assignment of the Raman bands in spectra of *B. subtilis*

Figure S4. SEM images of *B. subtilis* cultures after treatment at high temperature and acid condition

Figure S5. The effect of pH on the redox peaks of induced *B. subtilis* at room temperature

Table S2. Redox potential at different pH values

Figure S6. Peak currents for *B. subtilis* at pH=1.50 as a function of time

Figure S7. Cyclic voltammograms of supernatant

Figure S8. UPLC-Q-TOF analysis of standard NAD. MS spectra of NAD at m/z = 660-690 Da

Figure S9. DPV analysis of NAD redox substances
Transparent Methods

Bacterial Strain and Culture Conditions.
The strain used throughout this study was *Bacillus subtilis* GIMCC 1.258 (Microbial Culture Collection Center, Guangdong Institute of Microbiology, China). *B. subtilis* was grown in medium containing (g per liter): peptone, 10; NaCl, 5; yeast extract, 5; CuSO₄·5H₂O, 1.6; guaiacol, 1.6. The pH was adjusted to 4.68 and the medium was sterilized in autoclave for 20 min at 121°C. Cultures were carried out at 37 °C and 125 rpm in a rotary shaker.

Sample preparation for electrochemical analysis
*B. subtilis* was cultured for 7 days, and the culture broth was centrifuged at 5000 g for 15 min at 4°C. The supernatant was collected, and the pellet was washed three times with ultrapure water until the washing became colorless, ensuring the removal of any soluble redox species carried over from the culture media. The wet pellet (100 mg) was then resuspended in 1 mL ultrapure water for testing the electrochemical properties.

Thermostability tests
Samples were heated for 1, 3, 5, and 8 h at 26, 40, 60, 80, and 100°C using a water bath. After incubation, samples were centrifuged at 5000 g for 15 min at 26°C. The supernatant was kept 4°C until further analysis. The electrochemical activity of the pellets was analyzed by cyclic voltammetry.

Low pH stress tests
*B. subtilis* was cultured for 7 days, and the culture broth was centrifuged at 5000 g for 15 min at 4 °C. The supernatant was discarded, and the pellet was washed three times with ultrapure water until the washing became colorless, ensuring the removal of any soluble redox species carried over from the culture media. The wet pellet (100 mg) was then resuspended in 1 mL 0.20 M phosphate buffer (pH=1.50) and saved in 50mL glass tube sealed using a silica gel plug, and wrapped to keep dust from falling. The whole process
was performed in biosafety cabinets. Samples were sterilized in phosphate buffer (pH = 1.50) for 736 d. After incubation, samples were analysed for electrochemical activity using cyclic voltammetry.

**Electrochemical tests**

Cyclic voltammetry was performed using an Autolab 302N potentiostat (Metrohm Autolab, Utrecht, Netherlands). Three-electrode system was used for the experiments. Glassy carbon electrodes were used as working electrodes along with a platinum wire as the counter electrode and an Ag/AgCl reference electrode. 5 μL of *B. subtilis* suspension was placed on the glassy carbon surface and immobilized by 10 μL of Nafion ionomer (5% dispersion). The electrode was then placed in an oven at 60°C for 30~60s. Scans were performed at a rate of 10 mV s⁻¹, in 200 mM phosphate buffer. To confirm that redox peaks originated from reaction compounds, the supernatant was collected for subsequent differential pulse voltammetry (DPV). For the DPV study of oxidation reactions, the modulation amplitude was 0.035 V, the initial potential was -0.60 V and the final potential was +0.80 V. Chronoamperometry measurements were used to confirm that redox compounds are directly linked to *B. subtilis* metabolic activity. A three-electrode system was used for the analysis. During the testing, the working electrode was held at 0.15 and at 0.50 V for *B. subtilis*.

**SDS-PAGE**

Proteins were extracted from cells and spores and concentrated for loading onto the gel, using a total protein extraction kit and following the manufacturer’s instructions (KeyGen Biotech, Nanjing, China). Protein extracts were analyzed using 12% polyacrylamide resolving gels and 4% polyacrylamide stack gels. The loading quantity of sample was 10 μg. All samples were bathed in boiling water for 5 min and electrophoresis was run at a voltage of 100 V for 2.5 hours. The protein bands were visualized by Coomassie Brilliant Blue G-250 staining, methanol and acetic acid destaining.
Scanning electron microscopy (SEM).

Samples were fixed in 2.5% (w/v) glutaraldehyde for 6 h. After washing three times with phosphate buffer (200 mM, pH 4.68), the samples were dehydrated in an ethanol series (30%, 50%, 70%, 90%, and 100% twice). After supercritical drying for 6 h, the samples were placed onto conductive adhesive, and platinum was sprayed on the surface of the sample. SEM images were taken in a Hitachi S-4800 Scanning electron microscope.

Raman

Bacteria were collected by centrifugation at 6000 rpm for 5 min, washed three times using sterile distilled water, and the pellet was deposited on a clean glass slide. Raman spectra of B. subtilis were recorded on a Horiba Jobin Yvon S.A.S. LabRAM Aramis Laser confocal Microscope Raman spectrometer, with a 532 nm HeNe laser yielding 14 mW power at the sample, and an exposure time of 3 s.

UPLC-Q-TOF-MS

The mobile phase was a multistep linear solvent gradient of isopropanol (A) and methanol (B), as follows: 0-5 min: 25% A, 75% B; 3.1-6 min: 0% A, 100% B; 6.1-9 min: 25% A, 75% B; 9.1-12 min: 25% A, 75% B. The flow rate was 0.4mL min⁻¹ and the equilibration time was 12 min. The mass spectrometer operating conditions were as follows: Sample Cone 40 V; Source Offset, 80; Source temperature 100°C; Desolation temperature: 450 °C; Cone Gas, 0 L h⁻¹; Desolation Gas: 800 L h⁻¹; Capillary voltage: 3.0 kV. Spectra were recorded in positive ion mode scanning from 100 to 1000 Da.
Figure S1 (related to Figure 1). Cyclic voltammogram of *B. subtilis* treated with EDTA to chelate Fe$^{3+}$ to inactivate cytochrome c and inhibit activity.
Figure S2 (related to Figure 1). SDS-PAGE analysis of *B. subtilis* protein. M = marker, A = *B. subtilis*. The arrows show the position of a 38 kDa band attributable tocytochrome c.
Figure S3 (related to Figure 1). Raman spectra of pellet: a) *B. subtilis*; b) *B. subtilis* response after heat treatment at 100 °C for 5 h.
**Table S1 (related to Figure 1).** Assignment of the Raman bands in spectra of *B. subtilis*

| Band (cm\(^{-1}\)) | Assignment                                                        |
|---------------------|-------------------------------------------------------------------|
| 507                 | S-S disulfide stretching band                                      |
| 1219                | Amide III (arising from coupling of C-N stretching and N-H bonding) |
| 1330                | Phospholipids/DNA                                                 |
| 1469                | Lipids                                                            |
| 1545                | Amide II                                                          |
| 1583                | Cytochrome c                                                      |
Figure S4 (related to Figure 1 and Figure 3). SEM images of: a) *B. subtilis*; b) 100 °C treated *B. subtilis*; c) 100 °C and 0.05 mol/L H$_2$SO$_4$ treated *B. subtilis*; d) *B. subtilis* at pH=1.50 phosphate buffer for 736 days.
Figure S5 (related to Figure 3). The effect of pH on the redox peaks of *B. subtilis* at room temperature. The different pH values for tests were: a: 1.50; b: 3.00; c: 4.68; d: 7.00; e: 8.50; f: 10.60; and g: 12.00.
**Table S2 (related to Figure 3).** Redox potential at different pH values

| pH   | Oxidation peak 1 (V) | Reduction peak 1 (V) | Oxidation peak 2 (V) | Reduction peak 2 (V) |
|------|----------------------|----------------------|----------------------|----------------------|
| 1.50 | + 0.57               | +0.41                | + 0.12               | + 0.06               |
| 3.00 | + 0.44               | + 0.25               | 0.00                 | - 0.09               |
| 4.68 | + 0.39               | + 0.26               | + 0.09               | - 0.06               |
| 7.00 | + 0.25               | + 0.08               | nd                   | nd                   |
| 8.50 | + 0.17               | + 0.04               | nd                   | nd                   |
| 10.60| + 0.01               | - 0.08               | nd                   | nd                   |
| 12.00| nd[^a]               | nd[^a]               | nd[^a]               | nd[^a]               |

[^a] nd: not determined/detected
Figure S6 (related to Figure 3). Peak currents for *B. subtilis* at pH=1.50 as a function of time. a: oxidation current at +0.57 V; b: reduction current at +0.41 V.
Figure S7 (related to Figure 6). Cyclic voltammograms of supernatant: a) *B. subtilis*; b) *B. subtilis* after heat treatment at 100° C for 5 h; c) *B. subtilis* after heat treatment at 100° C and 0.05 mol L⁻¹ H₂SO₄ for 5 h.
Figure S8 (related to Figure 7). UPLC-Q-TOF analysis of standard NAD. MS spectra of NAD at m/z = 660-690 Da.
Figure S9 (related to Figure 8). DPV analysis of NAD redox substances. A) oxidation peak of NAD for 5 h at 26 °C, pH=4.68; B) oxidation peak of NAD in pH=1.50 PBS for 5 h. All results are from a minimum of three biological replicates. Experiments were performed under N₂(g).