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Electron communication of *Bacillus subtilis* in harsh environments

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SUMMARY

Elucidating the effect of harsh environments on the activities of microorganisms is important in revealing how microbes withstand un-favourable 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* (*B. 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* utilises an extracellular electron transfer (EET) as an electron communication pathway between *B. subtilis* and the environment that involves the cofactor nicotinamide adenine dinucleotide (NAD) as an essential participant to maintain viability. Elucidation of the capability of non-acidophilic and non-thermophilic strain to maintain viability under these extreme conditions could aid understanding of 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 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 ability of the cell 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 (Cournet et al., 2010), but the latter has not been studied in detail for bacteria at high temperatures and low pH conditions. The 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 bioelectrochemical systems (Chen, LX., 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 environment under extreme conditions.

*B. subtilis* is an aerobic, gram-positive bacterium, with an ample metabolic repertoire, and it 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 of communicate with 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 extracellular electron transfer when non-acidophilic and non-thermophilic B. subtilis is subjected to harsh conditions.

In this work, we analyzed the electrochemical activity of B. subtilis under two conditions: low pH and high temperatures. In a series of controlled experiments, we assessed the electrochemical activity of B. subtilis kept at pH=1.50 over two years, while in another series of experiments we evaluated the electrochemical activity of 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 B. subtilis, while 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 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. B. subtilis was incubated at 26 °C and 100 °C and its electrochemical activity was assessed by cyclic voltammetry (Figure. 1). Bare glassy carbon electrode showed no redox peaks (Figure. 1a), while oxidation peaks at +0.39 V and +0.09 V and reduction peaks at +0.26 V and -0.06 V were observed for B. subtilis incubated at 26 °C (Figure. 1b). Interestingly, significant redox currents were produced by B. subtilis after treatment at 100 °C for 3 h (Figure. 1c), 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 /proteins that retain electrochemical activity after being subjected to high temperatures (Figure.2B).
Figure 1. Cyclic voltammogram of *B. subtilis* subjected to different temperature treatments for 3 h. Curve a: bare glassy carbon electrode. Curve b: *B. subtilis* incubated at 26 °C. Curve c: *B. subtilis* incubated at 100 °C.
**Figure 2.** Effect of exposure time on the CV peak currents for *B. subtilis* treated at 100 °C. Graph A: 1): oxidation current at +0.09 V; 2): reduction current at -0.06 V. Graph B: 3): oxidation current at +0.39 V; 4): reduction current at +0.26 V.

**Redox activity in conditions at differing pH values**

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 *B. subtilis* at various pH values also. Experiments were carried out over a range of pH values in the range pH = 1.50-12.00. When *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 firstly use this condition for the electrochemical tests. At pH=4.68, redox peaks were observed at +0.39 V and +0.26 V and at +0.09 V and -0.06 V, while at 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 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 pH=4.68 buffer after electrochemical testing at pH 12.00, all of 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 *B. subtilis* subjected to extreme conditions occurred.
To investigate the long-term stability in acidic conditions, *B. subtilis* was suspended in pH=1.50 buffer for 736 d. 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 (Figure. 3 and Figure. S6). These data showed that *B. subtilis* redox ability remains stable even after very long term exposure to low pH conditions.

Figure 3. Cyclic voltammograms of *B.subtilis* at extreme acidic conditions for 736 days.

**Electron communication of *B. subtilis***.

Our data shows that *B. subtilis* is able to maintain the redox ability after treatment at high temperature and 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 of live bacteria. To this end, chronoamperometry was used to evaluate the effect of acetate as a substrate on *B. subtilis*, and only viable bacteria or spores can respond to acetate, the data will confirm live *B. subtilis* on current change.

Based on the CV data, potentials at +0.15 V and +0.50 V were chosen to perform chronoamperometry analysis. When the potential was held at +0.15 V, the current due to the oxidation reaction at +0.09 V will change when acetate was added to the system, *i.e.* we held +0.15 V to determine if the electron 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, this 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. 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 2120 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. In the B. subtilis-electrode system, the current measured at +0.50 V is
therefore ten times higher than that at +0.15 V. Compared to 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 therefore needs to be identified.
Figure 4. Current responses of *B. subtilis* to addition of 30 mM acetate in pH=4.68 phosphate buffer. (A) The potential for CA measurements (not stirred) is +0.15 V vs Ag/AgCl. (B) The potential for CA measurements (not stirred) is +0.50 V vs Ag/AgCl. The arrow indicates addition of 200 µL sodium acetate solution. (Insert: Current response of a bare electrode)

Lysozyme test was used for verify that the redox peaks comes from vegetative cells, spores or both. The spores are resistant to lysozyme, whereas vegetative cells are disrupted by the enzyme. Lysozyme doesn’t show any redox activity in this condition as shown in the Figure 5a. After lysozyme-treated the remnant of cell debris or contents of cellular materials enter into supernatant, their electrochemistry activity can not be detected, so the electrochemistry signal origin from *B. subtilis*. In Figure 5b, 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 redox activity of the spores, while the disappearance of the peaks at +0.39 V and +0.26 V indicates that these are originated by the redox activity of intact vegetative cells.
Figure 5. Cyclic voltammograms of a) lysozyme at bare glassy carbon electrode; b) lysozyme-treated B. subtilis supernatant

Figure 6. Cyclic voltammograms of pellets and supernatant. B. subtilis centrifuged at 5000 g for 10 min to obtain the pellets, then washed three times and the re-suspended pellets were then incubated for 5 hours at 26 °C: pellets (black), supernatant (red).

Identifying redox substances of B. subtilis
In order 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⁺, while the fragment ion at m/z 685.4349 corresponds to NAD⁺-H+Na. In Fig. 7B NAD⁺ ion intensity is slightly lower than Fig. 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 peak NAD⁺+Na and NAD⁺+Na+H, respectively. These results are consistent with the mass spectrum observed using NAD⁺ as standard (Figure. S8).
**Figure 7.** UPLC-Q-TOF-MS analysis of supernatants of: A) *B. subtilis* resuspended in ultrapure water for 5 hours at 26°C; B) *B. subtilis* resuspended in ultrapure water for 5 hours at 100 °C; C) *B. subtilis* resuspended in pH=1.50 buffer for 5 hours at 26 °C.

In order to confirm the participation of NAD\(^+\) in the electrochemical activity of *B. subtilis*, we performed differential pulse voltammetry tests in supernatants spiked with NAD\(^+\) standard (Figure. 8 and Figure. 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 suggests that NAD$^+$ is indeed involved in EET.

Figure 8. DPV analysis of redox substances. A) the oxidation peak of supernatants from *B. subtilis* resuspended for 5 h at 26 °C, pH=4.68, B) the oxidation peak of the supernatants from *B. subtilis* resuspended in pH=1.50 PBS for 5 h. All results are from a minimum of three biological replicates. Experiments were performed under N$_2$(g).

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$^+/\text{NADH}$, -0.06 V and +0.09 V being assigned to cytochrome $c$, and that the extracellular electron transfer in $B. \text{subtilis}$ involve 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 those involves the release of extracellular polymeric substances (EPS). EPS can be proteins, nucleic acids, lipids and 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 Fig. 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 Fe$^{3+}$ 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 $c$.

Heating can enhance molecular movements that 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; Rothschild and Mancinelli, 2001), resulting in the decline of the redox activity of cytochrome $c$. 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/acid treatment (Figure. S7), suggesting that a molecule with redox activity is secreted to the extracellular medium.

**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 present under extreme nutrient deprivation, they differentiate into spores, extremely resistant to potentially
damaging environmental conditions (Barney and Austin, 2017). In the case of low-pH stress, in order
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 in order to maintain internal pH homeostasis (Wilks et al.,
2009).

Electron communication pathway

In an attempt to elucidate electron transport mechanism, we performed experiments to identify the
redox molecule(s) involved in EET. B. subtilis can secret flavins (Morokutti et al., 2005), which have
been reported as electron transfer mediators for other bacteria e.g. Shewanella. But in this study, flavins
may not appear to make a significant contribution to electron transfer since the culture medium contains
Cu^{2+}, which is known to form a stable complex with flavin through d-π back donation and may make
inactive the redox reaction of flavin. When CuSO_{4} and guaiacol are added to LB medium, flavin was
not detected in our experiments.

Most energy-producing processes require coenzymes such as nicotinamide adenine dinucleotide
(NAD), a highly abundant cellular component of bacteria that participates in electron transfer during
oxidation-reduction reactions that 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 bacteria 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 small molecule heat-stable molecule (Chini et al., 2017). NAD a key class of cofactors serve
as essential electron donors or acceptors in all biological organisms (Liu et al., 2018), drive 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 exponential growth phase (Wos and Pollard, 2009). Secretion of NAD to the extracellular
milieu has been reported in diverse species, such as E. coli, R. Capsulatus (Ying, 2006) and
microorganisms in activated sludge (Wos and Pollard, 2009), as well as by wood-degrading fungi and
animal cell (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 extracellular electron transfer is essential due 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 need 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 table files and can
befound with this article online

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AUTHOR CONTRIBUTIONS

F. Z and L.X. C designed research and write the manuscript, L.X. C performed cultivation, C.L. C performed electrochemistry analyses. S.H. W performed proteome analyses. J. R. V, R. C. T. S, C.A.R revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

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Highlights

*B. subtilis* maintains electrochemical activity at pH=1.50 for over 2 years.

*B. subtilis* maintains electrochemical activity up to 100 °C for hours.

NAD acts an essential participant to electron communication of *B. subtilis*. 