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Electroactivity across the cell wall of Gram-positive bacteria

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A R T I C L E   I N F O

A B S T R A C T

The growing interest on sustainable biotechnological processes for the production of energy and industrial relevant organic compounds have increased the discovery of electroactive organisms (i.e. organisms that are able to exchange electrons with an electrode) and the characterization of their extracellular electron transfer mechanisms. While most of the knowledge on extracellular electron transfer processes came from studies on Gram-negative bacteria, less is known about the processes performed by Gram-positive bacteria. In contrast to Gram-negative bacteria, Gram-positive bacteria lack an outer-membrane and contain a thick cell wall, which were thought to prevent extracellular electron transfer. However, in the last decade, an increased number of Gram-positive bacteria have been found to perform extracellular electron transfer, and exchange electrons with an electrode. In this mini-review the current knowledge on the extracellular electron transfer processes performed by Gram-positive bacteria is introduced, emphasising their electroactive role in bioelectrochemical systems. Also, the existent information of the molecular processes by which these bacteria exchange electrons with an electrode is highlighted. This understanding is fundamental to advance the implementation of these organisms in sustainable biotechnological processes, either through modification of the systems or through genetic engineering, where the organisms can be optimized to become better catalysts.

Contents

1. Introduction

Electron transfer reactions are at the core of numerous biological processes, in particular in respiration. During respiration most microorganisms are able to convert biochemical energy into ATP. This usually involves a cascade of reactions where electrons are transferred, via several redox proteins, from an electron donor to an electron acceptor. Most forms of respiration involve a soluble compound as an electron acceptor (e.g. nitrate, oxygen, and sulfate), however there are others where solid compounds (e.g. metal oxides, electrodes) act as the electron acceptor [1]. In this case, the terminal electron acceptor is insoluble and cannot enter the cell, and the microorganisms must perform extracellular electron transfer (EET) to connect their electron transport chain to the solid electron acceptor [2,3]. Today, it is well recognized that the reduction

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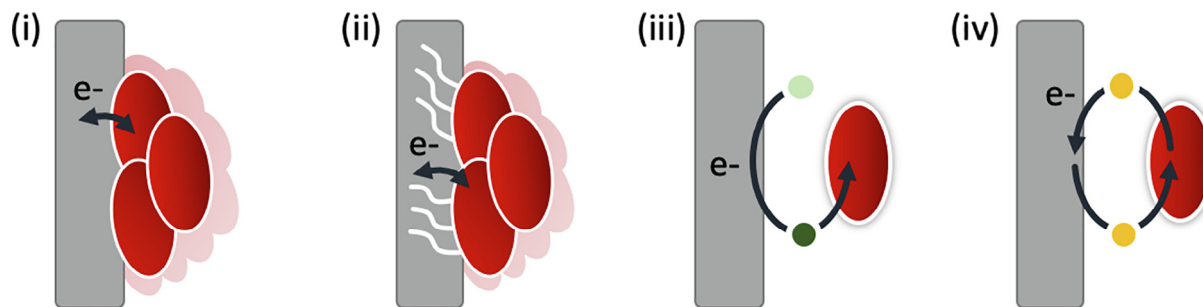   *E-mail address:* cpaquete@itqb.unl.pt

Fig. 1. Mechanisms of extracellular electron transfer (EET) processes. EET may occur through direct contact using cell-surface proteins, including multiheme *c*-type cytochrome (process i) or electrically conductive pilus (process ii), or through indirect electron transfer where chelators or siderophores solubilize the solid electron acceptor and transfer the electrons to the bacteria (process iii), or with soluble electron shuttles that mediate electron transfer between the cell and the solid electron acceptor (process iv). In this figure, the solid electron acceptor is represented in grey and bacteria are represented in red. Chelators/siderophores and electron shuttles are represented in green and in yellow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of solid electron acceptors occurs through two different mechanisms: directly, (i) through cell-surface proteins that interact with the solid electron acceptor (ii) or through cellular appendages, including electrically conductive pilus that form a bridge between the cell and the electron acceptor; or indirectly, (iii) through the use of chelators or siderophores that solubilize the solid electron acceptor and introduce them into the cell, or (iv) using soluble shuttles, such as organic compounds with quinones moieties, that interact with the electron acceptor outside of the cell [3–5] (Fig. 1).

Microorganisms that possess EET capabilities play a fundamental role in the geochemical cycle of several elements, including carbon and iron, and are also potential targets for numerous biotechnological applications, such as for the bioremediation of metal contaminated environments, production of energy and added-value compounds, or for biosensing [6–10]. Some of these organisms are also termed electroactive given their ability to exchange electrons with an electrode, in the so called bioelectrochemical systems (BES) [11,12]. Microbial fuel cells (MFC) are one of the most studied BES [13]. A typical MFC is an electrochemical cell arranged in two chambers separated by a proton exchange membrane, containing an anode and a cathode. In the anode compartment, the microorganisms oxidize organic matter and use the electrode as the terminal electron acceptor [6]. Typically, the electrons collected at the electrode are then transferred to the cathode through an external wire, and are combined with oxygen to generate water. The electron flow between the anode and cathode enables the electrical power harvesting [6,13]. The increased interest of this type of technology has boosted the application of BES, being currently explored for the production of electrical power, treatment of wastewaters, for electrosynthesis of added-value compounds and biofuels and for water desalination [14,15].

Electroactive organisms can be found in all three domains of life, being ubiquitous in distinct environments, including lakes, soils as well as in deep-sea hydrothermal vents [11,12]. Recently, is has been demonstrated that these organisms are also present in the human digestive system [16,17], in the mouse gut microbiome [18–20] and oral plaque [21], with some of them associated with infectious diseases [22,23].

Gram-negative mesophilic bacteria are one of the most studied class of electroactive organisms, with most of the knowledge being confined to the model organisms *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 [2,3,24]. Nonetheless, Gram-positive bacteria have recently attracted the scientific attention, given their capacity in producing high levels of current in MFC [25,26], and by being associated with infectious diseases in humans [16,17,22,23]. Given the importance of these organisms in BES, research has been dedicated in exploring their use as catalysts in BES and in the understanding of their EET processes [27–29]. Only

by understanding how electroactive organisms perform EET and exchange electrons to electrodes, it is possible to use them in biotechnological processes and start implementing BES in real-world applications. Indeed, knowledge on the molecular mechanisms of the EET processes performed by Gram-negative bacteria allowed to modify these organisms and improve their performance in BES [30–36], showing that synthetic biology field has the potential to advance the implementation of BES in the real-world [37].

As several reviews have been published on Gram-negative organisms [2,3,24], this review will focus on Gram-positive electroactive bacteria, in particular on the developments on the understanding of their EET mechanisms and on the cellular components involved in these processes.

2. Gram-positive electroactive bacteria

For several years Gram-positive bacteria were considered electrochemically inactive and EET was considered incompatible with this class of organisms [38,39]. In contrast to Gram-negative bacteria, Gram-positive bacteria lack an outer-membrane, contain a thick cell wall (20–80 nm) composed by peptidoglycan, teichoic acids, and sometimes are covered by a glycoprotein S layer [40,41], which were thought to prevent electron transfer to solid electron acceptors.

Nonetheless, when growing in co-cultures, some species of Gram-positive bacteria were showed to transfer electrons to an electrode in a MFC, being able to perform EET [39,42,43]. The first pure cultures of Gram-positive bacteria able to exhibit electrochemical activity were *Clostridium butyricum* [43], *Desulfitobacterium hafniense* [44], and *Lactococcus lactis* [45]. Studies on these organisms have demonstrated that Gram-positive organisms can perform indirect electron transfer, using electron shuttles excreted by them [45] or by other organisms [16,39,46]. But later, it was also shown that Gram-positive bacteria are able to perform direct electron transfer to electrodes, being this type of EET mechanism associated with a biofilm that is formed on the surface of the electrode [28,47,48]. Gram-positive bacteria can also perform EET by receiving electrons from a solid electron donor, including an electrode in microbial electrosynthesis (MES) [49,50]. These devices have recently attracted the interest of the scientific and industrial community, given the ability to couple microbial metabolism to the production of valuable chemicals and fuels, with the reduction of CO_2 [9].

Several electroactive Gram-positive bacteria are present as commensals in the intestines of numerous animals, whereas others are opportunistic pathogens [19,21,51,52]. Examples of these are *Lactococcus monocytogenes* [51], *Enterococcus faecalis* [52], *Entero-*

Table 1
List of Gram-positive bacteria described to be electroactive.

Year	Microorganism	Source	Reference
2001	*Clostridium butyricum* EG3	MFC containing starch processing wastewater	[43]
2006	*Brevibacillus* sp. PTH1	MFC containing sludge and domestic and industrial wastewater	[55]
2007	*Desulfitobacterium hafniense* strain DCB2	Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ)	[44]
2008	*Thermincola potens* strain JR	MFC containing sludge from thermophilic methanogenic anaerobic digester	[26]
2008	*Thermincola carboxydophila*	Sediment MFC containing marine marsh sediment	[56]
2009	*Bacillus subtilis*	Laboratory culture	[57]
2009	*Lactococcus lactis*	Meiji Milk Products Co., Ltd.	[45]
2009	*Thermincola ferriacetica*	DSMZ	[48]
2012	*Faecalibacterium prausnitzii*	DSMZ and human feces	[53]
2014	*Enterococcus faecalis*	MFC containing sludge	[58]
2014	*Clostridium pasterianum* DSM 525	DSMZ	[49]
2014	*Corynebacterium glutamicum*	American Type Culture Collection (ATCC)	[50]
2015	*Thermoanaerobacter pseudethanolicus*	ATCC	[25]
2016	*Clostridium beijerinckii* IB4	Mutant formed by ion implantation	[59]
2017	*Bacillus thuringiensis*	Laboratory culture	[60]
2018	*Listeria monocytogenes*	Unité des Interactions Bactéries Cellules laboratory's Listeria strain collection	[16]
2018	*Bacillus megaterium* strain LLD-1	MFC containing sludge from JiMei wastewater treatment plant	[61]
2019	*Bacillus cereus* DIF1	China Center for Type Culture Collection (CCTCC)	[62]
2019	*Rhodococcus ruber* DIF2	CCTCC	[62]
2019	*Clostridium cochlearium*	DSMZ	[18]
2020	*Paenibacillus dendritiformis* MA-72	Sediment MFC containing sediment from river Strum	[28]

coccus avium [21], *Clostridium cochlearium* [18] and *Faecalibacterium prausnitzii* [53,54]. Indeed, in several of them the presence of an EET system was shown to be important for the colonization of pathogenic bacteria [21,51].

Up to date more than 10 species of Gram-positive bacteria were identified as electroactive (Table 1).

When compared with Gram-negative bacteria, the EET mechanisms of Gram-positive bacteria have been less explored. This is mainly associated with the difficulties encountered during growth as a pure culture in the laboratory, the lack of their genetic information, and, in most cases, the impossibility of genetically manipulate these organisms. Up to now, the EET mechanism of Gram-positive bacteria were only explored for a few organisms, showing that Gram-positive bacteria can perform direct and indirect electron transfer to solid electron acceptors. Understanding the processes by which Gram-positive bacteria perform EET is of significant relevance to enhance electroactivity and optimize BES, either by modifying electrode materials and bioreactor set-up, or through genetic modification where electroactive organisms can be genetically engineered to become better than their counterpart wild-type strains in terms of electroactivity and/or metabolic functionalities [36]. The main mechanisms performed by Gram-positive are described below, focusing on the molecular processes by which these organisms perform EET.

3. Indirect electron transfer in Gram-positive bacteria

Indirect electron transfer to solid electron acceptors takes place in the presence of small redox active compounds, that mediate electron transfer between the microorganism and the solid electron acceptor, or vice-versa. These redox compounds can be a metabolite produced by the microorganism (e.g. flavins, phenazines, quinones) [62–64] or an artificial electron shuttle that can be added to the system (e.g. poly(MPC-co-VF), macrocyclic cobalt hexamines, osmium redox polymers) [57,65,66].

The electroactivity of Gram-positive bacteria was first associated with their ability in using electron shuttles produced by Gram-negative bacteria belonging to *Pseudomonas* sp. [46]. Indeed, metabolites produced by *Pseudomonas* sp. were responsible for the Gram-positive bacterium *Brevibacillus* sp. PTH1 to achieve EET [39]. But later on, it was demonstrated that Gram-positive bacteria can also produce redox mediators, such as humic acids, quinones and flavins [16,44,61,62,67]. For example, *Lactococcus lactis* cells can catalyse EET to an electrode by the excretion of soluble quinones as redox mediators [45], while spore-forming bacteria belonging to *Bacillus* genus have the ability to excrete flavins [41,61,62,68–70]. These flavins enable *Bacillus* sp. to mediate electron transfer to electrodes in MFC [62,68], and provide a boost to electricity generation in microbial consortia with Gram-negative bacteria or yeasts [60,69,70]. Recently, it was also shown that flavins are secreted by *Rhodococcus ruber* DIF2, and that flavin mononucleotide (FMN) plays an important role in the EET of this bacterium to electrodes [62]. Though humic acids were shown to support the generation of electricity of *Desulfitobacterium hafniense* strain DCB2, the specific electron shuttle responsible to mediate electron transfer to the electrode was not identified [44].

The cofactor nicotinamide adenine dinucleotide (NAD) was also shown to play a significant role in the viability and electroactivity of *B. subtilis* when submitted to long-term exposure to harsh environments [71]. In these conditions *B. subtilis* can use EET as an electron communication pathway, where NAD is an essential participant to maintain its viability [71].

The incorporation of *Bacillus* cells in an anaerobic sludge also had a significant effect in the power generation in a MFC [72]. By promoting the formation of an electroactive biofilm and by supressing methanogenesis, *Bacillus cereus* enhanced current production in MFC [72]. Furthermore, a genetically modified strain of *B. subtilis* RH33 that produces high levels of riboflavin were also shown to boost electricity generation in a microbial consortium with *S. oneidensis* MR-1 [70], emphasising the importance of indirect electron transfer in EET of Gram-positive bacteria.

The electroactivity of *Listeria monocytogenes* has been observed almost three decades ago [73], but only recently it was demonstrated that this Gram-positive bacterium can use environmental flavins to shuttle electrons to solid electron acceptors [16]. An eight-gene locus was found to be responsible for the EET capacity, with a NADH dehydrogenase channelling electrons to a membrane-localized quinone pool, and an extracellular flavoprotein that, in conjunction with flavins, mediate electron transfer to extracellular acceptors [16]. This locus is present in numerous organisms within the Firmicutes class of organisms, which suggest that the flavin-based transfer mechanisms is highly conserved in Gram-positive bacteria [16,51]. Indeed, this gene cluster was found to be important for EET to ferric iron in *E. faecalis* [22], indicating that the proteins involved in this process are similar in Gram-positive bacteria. It was proposed that an atypical NADH hydrogenase (NDH-3 in *E. faecalis* and NDH-2 in *L. monocytogenes*) couple the oxidation of NADH in the cytoplasm to reduction of demethyl-menaquinone (DMK) (Fig. 2A). Given that DMK is the only quinone available in the membrane of *E. faecalis*, it can either be oxidised by cytochrome *bd* under aerobic conditions and in the presence of
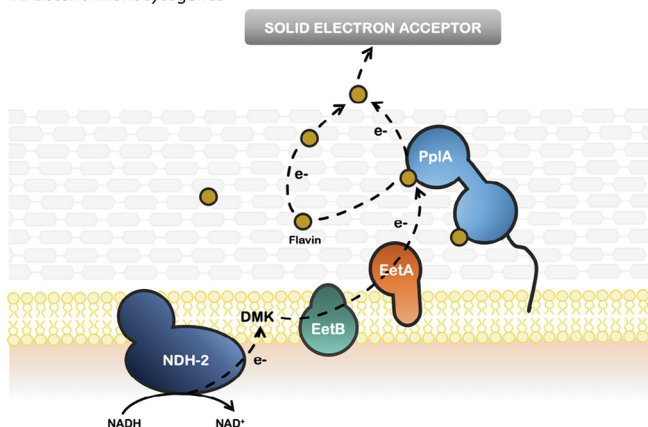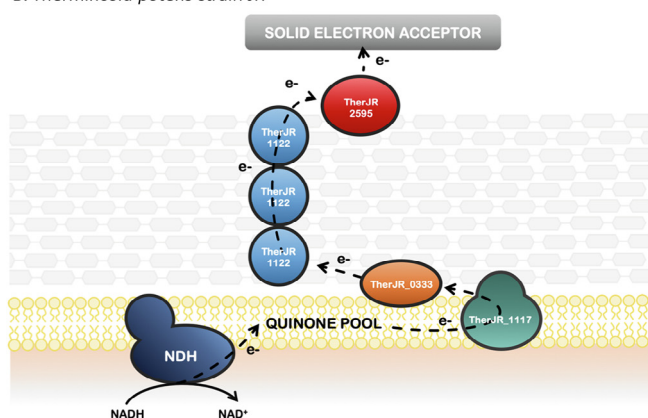
A. *Listeria monocytogenes*

B. *Thermincola potens* strain JR

Fig. 2. Model for the proposed EET processes pathway of *L. monocytogenes* (A) (adapted from [16]) and *T. potens* (B) (adapted from [29]).

heme, or under anaerobic environments by EET processes [22,52]. The lipoprotein PplA present at the surface of *L. monocytogenes*, was shown to bind FMN, being proposed to be an important component in the EET pathway of this Gram-positive bacterium, facilitating electron transfer between FMN and extracellular electron acceptors [16,22]. Two small proteins, EetA and EetB were also found to be important for EET [16,22]. EetA was predicted to be a membrane protein anchored to the outer-side of the cytoplasmic membrane, while EetB was demonstrated to be an integral membrane protein that contains four transmembrane segments and a large periplasmic loop (Fig. 2A). Although it was proposed that these proteins form a complex in the membrane their role remains to be elucidated [22]. Hederstedt and co-workers proposed that when osmium complex-modified redox polymers (OsRP) are used as mediators, *E. faecalis* uses a different EET pathway. This pathway does not depend on the NADH dehydrogenase and on EatA, suggesting that OsRP receives electrons directly from the DMK [22].

The use of siderophores has also been observed in several electroactive Gram-positive bacteria [74–76]. In *C. ferrireducens*, a supplementary strategy for the utilization of siderophores in the reduction of iron oxides has been proposed [74]. This suggests that, as in Gram-negative bacteria [77–79], different EET pathways exist in Gram-positive bacteria, and that these may depend on the environment and growth conditions.

4. Direct electron transfer in Gram-positive bacteria

Most electroactive organisms use both types of EET processes to exchange electrons with an electrode, which make it difficult to identify solely the direct electron transfer process. Evidence of this type of mechanism is usually assessed by the formation of a biofilm, and by the lack of impact in current production after removing the medium or after adding redox mediators [47,48].

Direct electron transfer to an electrode by Gram-positive bacteria was first identified in *Thermincola* sp. [48]. *T. ferriacetica* was capable of generating an electric current in an air–cathode MFC without the addition of soluble redox mediators [48]. The lack of electron shuttles, the formation of a biofilm on the electrode and the rapid current recovery by this strain after exchanging the media were shown to be consistent with direct EET. Furthermore, the Coulombic efficiency observed for this organism was higher than 95%, indicating that nearly all electrons were used for electrode reduction and not for the production of reduced organic compounds [48]. This behaviour was also observed for *T. potens* strain JR, suggesting that direct EET is characteristic of this genus [47]. In contrast to *T. ferriacetica*, *T. potens* strain JR could use soluble electron shuttles to transfer electrons to solid electron acceptors, although no soluble redox mediators have been identified in the MFC spent medium [47].

Genomic analysis of *T. potens* strain JR [80] have led to the proposal that multiheme *c*-type cytochromes (MHC) could be involved in EET in Gram-positive bacteria, as observed for Gram-negative electroactive bacteria. Indeed, trypsin-shaving LC-MS/MS experiments and surface-enhanced Raman spectroscopy allowed the identification of several MHC that could be involved in EET in this organism [29]. Although the identification of *c*-type cytochromes as key proteins for the reduction of insoluble electron acceptors has been previously observed for Gram-positive bacteria [74], this was the first time that an EET pathway was proposed for the transfer of electrons outside of the cell [29]. The proposed EET pathway is composed by four MHC, that were shown to be conserved among *Thermincola* sp. [81], suggesting that a general strategy for electron transfer may occur within this genus (Fig. 2B). As observed for several Gram-negative bacteria, the EET pathway in *Thermincola* sp. is composed by an inner-membrane MHC that receive electrons from the menaquinone pool, that then transfers the electrons to a periplasmic cytochrome. The transfer of electrons outside of the cell depends on a hexaheme cytochrome that was proposed to be embedded in the peptidoglycan at the cell wall and on the nine-heme cytochrome OcwA present at the cell-surface of these bacteria, responsible to reduce solid electron acceptors, electron shuttles and oxyanions [27,29] (Fig. 2B). This arrangement is different from what is typical observed in electroactive Gram-negative bacteria, where porin-cytochrome complexes composed by a β-barrel porin and one or two MHC are responsible for electron transfer across the outer membrane [82].

Direct electron transfer was also observed for other Gram-positive bacteria including *Chlostridium pasteurianum* [49], *P. dendritiformis* MA-72 [28] and *Carboxydothermus ferrireducens* [74], although the molecular processes and the proteins involved are still unknown.

In Gram-negative bacteria, direct electron transfer was also observed to occur through electrically conductive pili [83] or outer membrane extensions [84]. These structures allow the microorganism to make an electrical connection between periplasmic carriers and the insoluble electron acceptors, that can be located as far as 15 μm away from the cell [85].

Filaments of the type IV pili were also reported for some Gram-positive bacteria [86]. Although most of the functions of type IV pili in Gram-positive bacteria have been associated with mobility and adherence to host cells [86,87], pili-like appendages have been observed in *C. ferrireducens* grown on iron oxides [74], in *Paenibacillus dendritiformis* when growing on an electrode [28] and in *E. faecalis* pili revealed to contribute for EET [88]. It was demonstrated that *E. faecalis* biofilm matrix harboring iron sinks promotes

EET and augments biofilm growth, increasing current generation in MFC [89]. The biofilm associated pilus (Ebp) was demonstrated to be a key player in this process by sequestering iron in close proximity to the cells, either as surface attached pili or within the biofilm matrix, enabling *E. faecalis* to use it as terminal electron acceptor [88].

Extracellular polymeric substances (EPS) surrounding electroactive organisms help cells to attach to solid minerals or surfaces, assisting biofilm formation and protecting them from unfavourable environments [90]. EPS were also demonstrated to have electroactive properties, due to the presence of nucleic acids, humic substances, flavins and even proteins that are redox-active or semiconductive [91,92]. EPS from *Bacillus* sp. WS-XY1 was shown to be electroactive and of significant importance for EET in this bacterium [92].

5. Summary and outlook

EET between microbes and solid electron acceptors/donors, such as iron minerals or electrodes, is a widespread process that affect biogeochemical cycles, microbial ecology and that can be explored for the generation of electricity and chemicals in BES. For this reason, the elucidation of the mechanisms by which microorganisms perform electron transfer between intracellular and extracellular environments has been subject to widespread attention. Although most of electroactive organisms discovered to date are Gram-negative bacteria [11,12], Gram-positive bacteria were also demonstrated to perform EET, being able to transfer electrons to solid compounds directly and indirectly. Like Gram-negative bacteria, some Gram-positive bacteria rely on MHC to transfer electrons to solid electron acceptors outside of the cell. Up to date the only cell-surface cytochrome from Gram-positive bacteria that have been characterized in detailed was the OcwA from *T. potens*, showing that it can reduce solid electron acceptors, soluble electron shuttles and oxyanions [27]. Nevertheless, the way this protein is anchored and arranged at the cell surface and how electrons are transferred across the cell wall are still to be determined. In the fermentative Gram-positive bacteria *L. monocytogenes* and *E. faecalis* flavin-interacting proteins represent the extracellular components of their EET machinery, facilitating electron transfer, via FMN, to extracellular electron acceptors [16]. Although a cluster of proteins has been proposed to be widespread within the Firmicutes phylum, and to be important for EET, the mechanisms by which these organisms exchange electrons with solid electron acceptors remains to be elucidated.

The recent studies on Gram-positive bacteria revealed that, as in Gram-negative bacteria, a diverse way to perform EET exists in this class of microorganisms, including direct and indirect electron transfer. This demonstrates that rather than being a specialized process, EET is a fundamental process of microbial metabolism that occurs in numerous organisms and across diverse environments. The characterization of these processes is crucial not only to unravel the involvement of microbial metabolism in the biogeochemical cycle of elements, but also to enhance the development of novel sustainable biotechnological processes, where these organisms can be employed. Indeed, although Gram-positive bacteria can be more sensitive to several compounds (e.g. antibiotics, cleaning agents) given the high permeability of the peptidoglycan layer, their thick cell wall in addition to their ability in forming spores, enables their use in more extreme conditions [18,21,26].

Although knowledge of the EET performed by Gram-positive bacteria have increased in the last years, much more studies are required to fully understand their molecular processes in exchanging electrons with solid electron acceptors or donors. As future work, studies on the proteins involved in these processes, as well as the understanding of their electron transfer mechanisms should be a priority. Only with this knowledge it will be possible to improve these organisms and boost their performance beyond their natural metabolic capabilities, allowing to advance the implementation of BES and expand their biotechnological application.

CRediT authorship contribution statement

Catarina M. Paquete: Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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