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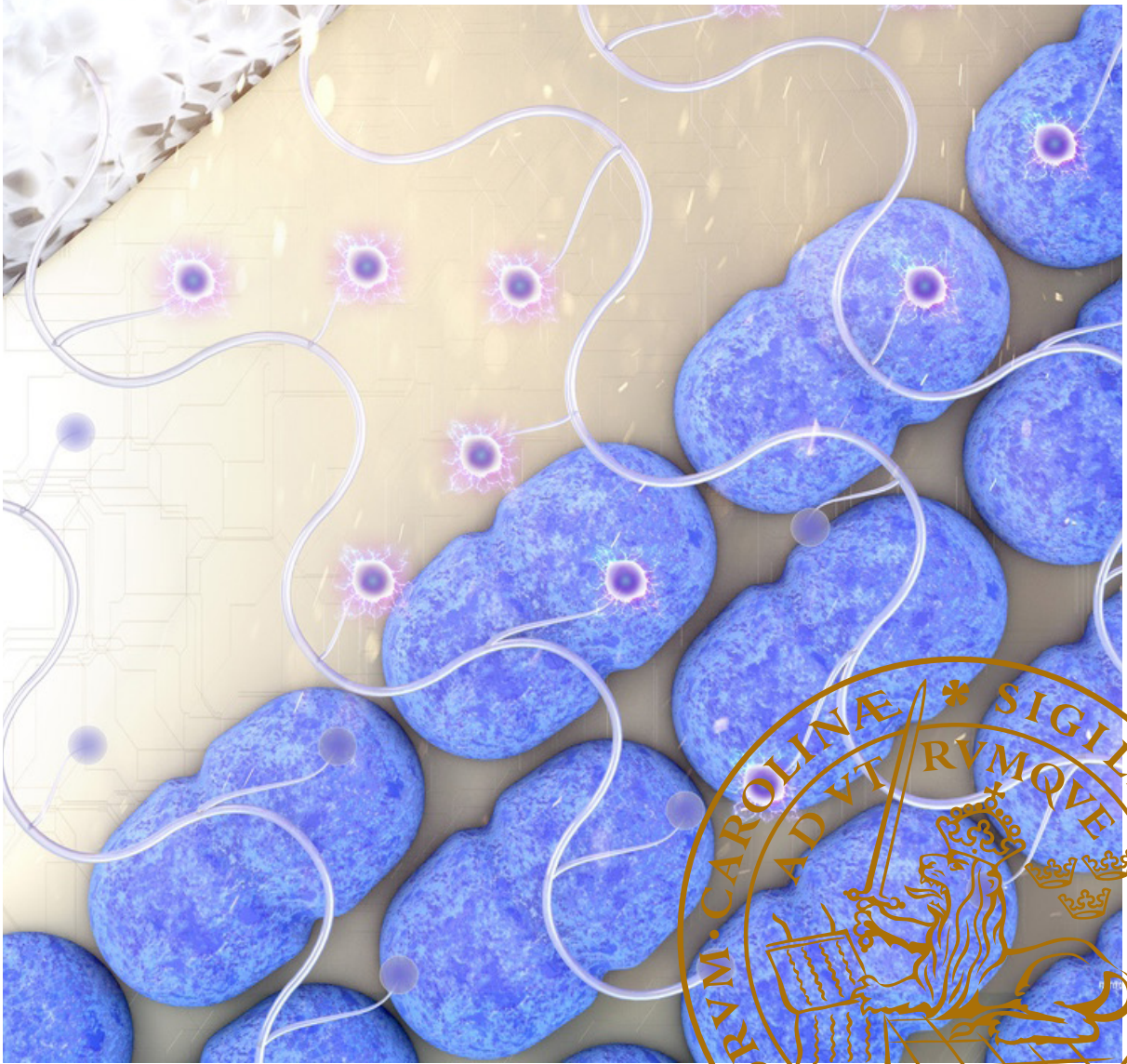

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Extracellular electron transfer features of the Gram-positive bacterium *Enterococcus faecalis*

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Abstract

Electroactive microorganisms possess the unique ability to transfer electrons to or from a solid phase electron conductor, e.g., electrodes or minerals, through various physiological mechanisms. The processes are commonly known as extracellular electron transfer and broadly harnessed in microbial electrochemical systems, such as microbial electrosynthesis or microbial fuel cells. Except for a few model microorganisms, the nature of the microbe-electrode conductive interaction is poorly understood for most of the electroactive species, which determines the efficiency and the potential scaling up of bioelectrochemical systems. The use of electron transfer mediators, such as electron-conducting polymers, is considered as one of the promising strategies to enhance the electron transfer efficiency up to the scale of a real life application.

Gram-positive bacteria are microorganisms carrying a thick electron non-conductive cell wall and believed to exhibit weak extracellular electron shuttling activity. Most of the reported cases are barely understood and offer poor and frivolous knowledge about the electron transfer and associated mechanisms in Gram-positive bacteria. Far less is known about the electron transfer of the bacteria mediated by high molecular weight redox polymers.

This thesis presents studies on the extracellular electron transfer mechanisms and properties of the Gram-positive bacterium *Enterococcus faecalis.* The bacterium was confirmed to perform extracellular electron transfer both directly and via mediators. The wild-type strain and some mutants were investigated electrochemically in combination with various mediating compounds. The *E. faecalis* cells are demonstrated to transfer electrons to electrode surfaces via a demethylmenaquinone pool in the membrane. Heme proteins presented in the cells under aerobic conditions are shown to hinder such electron transfer processes.

Furthermore, the cell-polymer-electrode mediated interaction through osmium and quinone-based polymeric mediators is characterized in detail. The obtained experimental data suggest that a redox polymer can be directly incorporated into the respiratory chain of the bacterial cells for accepting electrons and to further shuttle them to the electrode surface. The attained results will help in further development and adaptation of mediators for microbial-based systems.

The findings may add an essential piece of fundamental understanding of the nature and possibilities of microbial extracellular electron transfer, and advance our knowledge in interspecies electron transfer and the cycling of biogeochemical elements in nature. Additionally, a comprehensive understanding of cell-electrode interactions may help in overcoming insufficient electron transfer and restricted operational performance of various bioelectrochemical systems.

Key words: Gram-positive bacteria, *Enterococcus faecalis*, extracellular electron transfer, electron-conducting redox polymers, mediated electron transfer.

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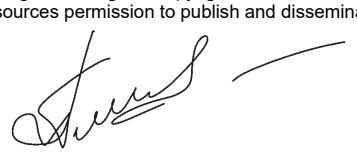
Signature  **Date** 2018-10-15

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MADE IN SWEDEN

To my beloved parents

“But I don’t want to go among mad people,” Alice remarked
“Oh, you can’t help that,” said the Cat:
“we’re all mad here. I’m mad. You’re mad.”
“How do you know I’m mad?” said Alice.
“You must be,” said the Cat, “or you wouldn’t have come here.”

Lewis Carroll, Alice’s Adventures in Wonderland

Contents

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Electrochemical communication between living cells and conductive surfaces**.
G. Pankratova, L. Gorton. *Current Opinion in Electrochemistry* 2017, 5, 193-202.

II. **Electrochemical wiring of the Gram-positive bacterium *Enterococcus faecalis* with osmium redox polymer modified electrodes**.
G. Pankratova, K. Hasan, D. Leech, L. Hederstedt, L. Gorton. *Electrochemistry Communications* 2017, 75, 56-59.

III. **Electron transfer between the Gram‑positive *Enterococcus faecalis* bacterium and electrode surface through osmium redox polymers**.
G. Pankratova, E. Szypulska, D. Pankratov, D. Leech, L. Gorton. *ChemElectroChem* 2018, DOI: 10.1002/celc.201800683.

IV. **Extracellular electron transfer by the Gram-positive bacterium *Enterococcus faecalis***.
G. Pankratova, D. Leech, L. Gorton, L. Hederstedt. *Biochemistry* 2018, 57, 4597-4603.

V. **Following nature: bioinspired mediation strategy for Gram-positive bacterial cells.**
G. Pankratova, D. Pankratov, R. D. Milton, S. D. Minteer, L. Gorton. Manuscript submitted.

My contribution to the papers

Paper I. I performed a literature review and took a large part in writing and revising the manuscript.

Paper II. I took part in planning the experiments, performed the experimental work and data interpretation, wrote the first draft of the manuscript and took part in revising the manuscript .

Paper III. I designed the experimental work and trained E. Szypulska to conduct the experiments, took part in evaluation and interpretation of the results, wrote the first draft and took part revising the manuscript.

Paper IV. I took part in planning the experiments, conducted all electrochemical experiments, assembled data, and took part in interpretation of the results, writing and revising the manuscript.

Paper V. I took part in planning the experiments, performed the experimental work, took part in data interpretation and evaluation, and wrote the first draft of the manuscript.

Other publications not included in the thesis

I. **Wiring of photosystem I and hydrogenase on an electrode for photoelectrochemical H_2 production using redox polymers for relatively positive onset potential**.
C. Tapia, R. D. Milton, G. Pankratova, S. D. Minteer, H.-E. Åkerlund, D. Leech, A. L. de Lacey, M. Pita, L. Gorton. *ChemElectroChem* 2017, 4, 90-95.

II. **Supercapacitive photobioanodes and bio-solar cells: a novel approach for solar energy harnessing**.
G. Pankratova, D. Pankratov, K. Hasan, H.-E. Åkerlund, P.-Å. Albertsson, D. Leech, S. Shleev, L. Gorton. *Advanced Energy Materials* 2017, 7, 1602285.

III. **Supercapacitive biosolar cell driven by direct electron transfer between photosynthetic membranes and CNT networks with enhanced performance.**
D. Pankratov, G. Pankratova, T. P. Dyachkova, P. Falkman, H.-E. Åkerlund, M. D. Toscano, Q. Chi, L. Gorton. *ACS Energy Letters* 2017, 2, 2635-2639.

IV. **Three-dimensional graphene matrix supported and thylakoid membranes based high-performance bioelectrochemical solar cell**.
G. Pankratova, D. Pankratov, C. Di Bari, A. Goñi-Urtiaga, M. D. Toscano, Q. Chi, M. Pita, L. Gorton, A. L. De Lacey. *ACS Applied Energy Materials* 2018, 1, 319-323.

V. **Sunlight photocurrent generation from thylakoid membranes on gold nanoparticle modified screen-printed electrodes.**
H. Kanso, G. Pankratova, P. Bollella, D. Leech, D. Hernandez, L. Gorton. *Journal of Electroanalytical Chemistry* 2018, 816, 259-264.

VI. **Micropatterned carbon-on-quartz electrode chips for photocurrent generation from thylakoid membranes.**
A.-I. Bunea, A. Heiskanen, G. Pankratova, G. Tesei, M. Lund, H.-E. Åkerlund, D. Leech, N. Larsen, S. Keller, L. Gorton, J. Emnéus. *ACS Applied Energy Materials* 2018, 1, 3313-3322.

VII. **Substrate preference pattern of *Agaricus meleagris* pyranose dehydrogenase evaluated througt bioelectrochemical flow injection amperometry.**
P. Rafighi, P. Bollella, G. Pankratova, C. K. Peterbauer, P. Ó Conghaile, D. Leech, B. Haghighi, L. Gorton. *ChemElectroChem* 2018, DOI: 10.1002/celc.201801194.

VIII. **Supercapacitor/biofuel cell hybrid device employing biomolecules for energy conversion and charge storage.**
F. Shen, D. Pankratov, G. Pankratova, M. D. Toscano, J. Zhang, J. Ulstrup, Q. Chi, L. Gorton. Submitted manuscript.

List of abbreviations and symbols

ATP	Adenosine triphosphate
CE	Counter (auxillary) electrode
CPE	Constant phase element
CV	Cyclic voltammogram
D-Ala	D-Alanine
DET	Direct electron transfer
D-iso-Glu	D-Isoglutamine
DMK	Demethylmenaquinone
DNA	Deoxyribonucleic acid
EDL	Electrical double layer
EET	Extracellular electron transfer
EIS	Electrochemical impedance spectroscopy
ET	Electron transfer
FAD	Flavin adenine dinucleotide
FIA	Flow injection analysis
FMN	Flavin mononucleotide
L-Ala	L-Alanine
L-Lys	L-Lysine
MES	Microbial electrochemical system
MET	Mediated electron transfer
MFC	Microbial fuel cell
NADH	Nicotinamide adenine dinucleotide reduced
NAG	N-acetylglucosamine

NAM	N-acetylmuramic acid
OCP	Open circuit potential
RE	Reference electrode
RP	Redox polymer
SHE	Standard hydrogen electrode
sRNA	Small ribonucleic acid
WE	Working electrode

A	Electrode surface area
C	Concentration
	Capacitor in equivalent circuit schemes
C_{dl}	Double layer capacitance
E	Potential
f	Frequency of the potential perturbation
F	Faraday constant (~$9.6485 \cdot 10^4$ C mol^{-1})
I	Current
j	Imaginary unit
k^0	Heterogeneous rate constant
n	Number of electrons involved into one reaction step
R	Gas constant (~8.314 J mol^{-1} K^{-1})
	Resistor in equivalent circuit schemes
R_{ct}	Charge transfer resistance
R_s	Solution resistance
Z'	Real part of impedance
Z''	Imaginary part of impedance

Introduction

Microbial electrochemistry is a multidisciplinary research platform focusing on the study of electrochemical properties of microorganisms and their application in the interactions with electron conductors driven by extracellular electron transfer (EET) [1]. EET is a conductive communication, which allows electron transfer (ET) between cell metabolism and external solid materials, i.e., electrodes or minerals.

Microbial species, which are able to undergo such EET processes, are key drivers in global geochemical cycles of elements [2] and potential partners of electronically connected microbial communities [3]. Furthermore, the microbes performing the phenomenal external ET are currently of considerable attention due to their various biotechnological applications, e.g., recovery and bioremediation of metals, microbial electrosynthesis, and microbial fuel cells (MFCs) [4]. However, at the present time they are locked mostly within research level projects [5]. All microbial electrochemical systems are governed by electrode-microbe interactions, which are in turn strongly defined by the EET properties of the particularly involved biocatalyst.

Numerous microorganisms possessing the ability for electron exchange with solid surfaces directly or via mediators are assigned as electroactive, but intensive research was mostly focused on a limited group of strong electroactive species belonging to Proteobacteria (e. g., the genus *Shewanella* and *Geobacter*) [2, 6]. A recent comprehensive study [7] has revealed a wide phylogenetical diversity of electroactive microbial species, which include also various Firmicutes. Gram-positive bacteria are believed to be weak electricigens due to the cell envelope architecture [8], however, they are commonly found in MFCs [9, 10] and their role in such communities is not identified yet. However, a synergistic effect, i.e., a long-term power production and an increased stability, has been reported for various biotechnological applications employing mixed consortia including Gram-positive species [11-13].

The use of mixed microbial communities is a more feasible and sustainable approach in terms of real-life applications. However, the EET network is more complex in mixed biofilms. In order to assure rational development and optimization of the mixed microbial associations, the EET properties of each member have to be taken into account. Consequently, the key step to advance and

scale-up microbial electrochemical technologies is a comprehensive understanding of microbial EET mechanisms and pathways [4, 6].

Gram-positive bacteria inhabiting MFCs are potential electricity generators, however, a mediator supplement is required. In this case electron-conducting redox polymers (RPs) are a reasonable choice among other mediators to enhance the performance of the devices and advance scaling-up under mediated ET conditions [14]. However, an electronic communication between RPs and bacterial cells carrying a thick cell wall has been established only for a limited number of microorganisms [15, 16]. The mechanistic description of such interactions remains unclear, which limits the development of the PR design and their wide employment in microbial electrochemical technologies. Most studies in microbial electrochemistry, however, refer to Gram-negative bacteria. The electroactive Gram-positive bacteria in general are insufficiently understood, and the involved EET mechanisms are poorly studied [17].

This thesis is focused on the EET mechanisms and properties of the Gram-positive bacterium *Enterococcus faecalis*, an opportunistic pathogen living in the intestinal tracts of mammals. The underlying mechanisms of the electronic interaction between the bacterium and various mediators, including high molecular RPs have also been characterized.

These findings add an essential piece of fundamental understanding of microbial ET properties of Gram-positive bacteria and electronic synergetic cooperation of microorganisms in MFCs communities. The mechanistic description of the RP-cell electrochemical communication is crucial for development and employment of new mediators in microbial electrochemical systems (MES). Furthermore, the gained knowledge might give a new insight into the bacterial syntrophic metabolism in microbiota of mammals.

1. Extracellular electron transfer

ET reactions are fundamental processes in nature. The electronic coupling of microbial metabolism to an external solid surface, e.g., minerals or electrodes, leads to EET. The mechanisms of microbial EET are of high scientific interests, since knowledge and understanding of these are essential for environmental engineering technologies, biogeochemistry, microbial physiology and ecology [4, 6]. One can assume that microorganisms possessing a high electron discharge capacity are potentially electrochemically active. However, it should be noted that the phylogenetical and physiological diversity of the reported microorganisms and the way how the electroactivity is realized by each of the organisms challenge finding a defined description of microbial electroactivity [17]. EET is a complex phenomenon and depending on the mechanisms involved, the distances involved in microbial EET may vary greatly from the nanometer-scale, in case of ET across the cell envelope, to in excess of a centimeter-length for cable bacteria [18]. Generally, a metabolically active biocatalyst can exchange electrons with solid conductive surfaces using two main different routes: through direct ET (DET) and mediated ET (MET).

1.1. Direct electron transfer

DET occurs through a direct physical contact between the cell and an electrode without involvement of any diffusable redox compounds (Figure 1). It can be achieved if a microbe holds redox proteins in the cell membrane or cell envelope, e.g., cytochromes [19, 20], flavoproteins [21] or multi-copper proteins [22], which are able to allow transport of electrons between the inside of the cell and an external solid surface. In the case of only a thin layer of cells, which are in closest physical contact with the electrode surface, these may carry out EET [23]. Thus, the efficiency is limited by the maximum cell density in the bacterial monolayer. Some bacterial species, e.g., *Geobacter*, can establish long-range DET through conductive cell appendages, nanowires or pili [23, 24], which conduct electrons from distant internal layers of the biofilm over a distance of up to 10 μm. This allows development of a thicker electrochemically active bacterial multilayer and a higher ET yield. The molecular structure of nanowires has not yet been

completely experimentally defined [2], and the mechanism by which electrons are transferred along a pilus is not completely understood. The main proposed models of conductance in nanowires are multistep electron hopping [25] and metal-like conductive mechanisms [26-29].

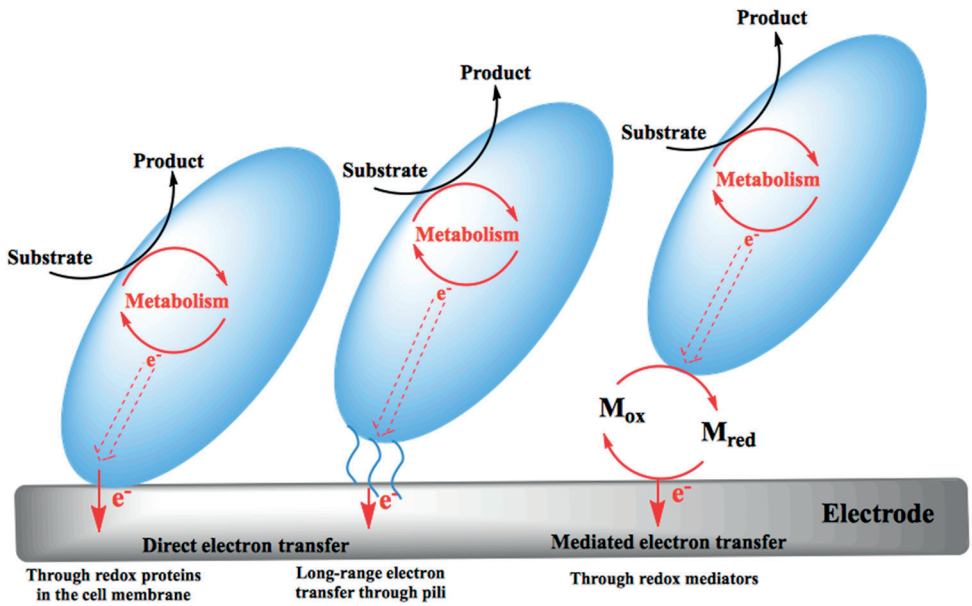


Figure 1. Schematic representation of anodic EET mechanisms.

Direct interspecies ET is a direct cell-to-cell transfer of electrons through physical connection between different microbial species. According to those studies reported so far, three different mechanisms accomplishing this type of electrical connection have been proposed [30]: (i) ET through electrically conductive pili [31, 32], (ii) ET between redox proteins bound to the outer cell surfaces [33], or (iii) ET through abiotic conductive materials [34, 35]. None of the listed strategies have been studied in deep detail; many questions are still to be answered to further resolve the mechanisms of direct interspecies ET.

Furthermore, a novel cell-to-cell electrical connection has been proposed for sediment living cable bacteria from the family Desulfobulbaceae. These bacteria form multicellular cables of thousands of cells and are supposed to accomplish intercellular ET across the whole filament over exceptional centimeter-scale distances by connecting reduction of oxygen and oxidation of sulfides at the terminal end of the cell chain [36, 37]. The molecular mechanisms of this extraordinary long-range ET are unknown.

1.2. Mediated electron transfer

MET takes place by the presence of redox active mediating compounds, which shuttle electrons between a microorganism and an external donor/acceptor (Figure 1). MET can be realized in a numbers of ways depending on the nature and the source of the mediating redox species, which can be an artificial mediator added to the system or metabolites secreted by a microbe. Bacteria produce various primary and secondary metabolites, which may be involved in EET [38]. Certain microbial species produce various diffusible reduced primary metabolites, e.g., H_2 [39, 40] and H_2S [41], which can be oxidized at the electrode surface generating an electron flow. Endogenous low-molecular weight mediators, secondary metabolites, are secreted by several bacterial species, e.g., phenazines by *Pseudomonas aeruginosa* [13, 42], flavins by *Shewanella oniedensis* [43, 44] and quinones by *Lactococcus lactis* [45]. With respect to anodic processes, a natural diffusible mediator can be reduced with the aid of electrons released in the bacterial cytoplasmic membrane and diffuse to the anode to be reoxidized at the electrode surface and, thus, deliver electrons. Such a shuttling activity brings deeper cell layers of a biofilm in contact with the electrode surface [46]. The process is reversible and the mediating species can undergo redox cycling many times. Thus, even a small amount of excreted redox active metabolites can facilitate ET. Furthermore, a mediator excreted by one microbe may support ET from another microbial species in the biofilm. This synergetic interaction increases the efficiency of current generation in microbial consortia [9].

Microorganisms generally have a cell envelope composed of essentially non-electron conductive materials like peptidoglycan, lipopolysaccharides, and lipid bilayer membranes preventing DET. Externally provided artificial mediators are thought to overcome this limitation and effectively link the microbial metabolism to an electrode enabling EET [47]. However, the efficiency may vary depending on the nature of the redox mediator. An ideal mediator should meet certain requirements: good solubility, long-term stability both in its oxidized and reduced forms, non-biodegradability, non-toxicity to microorganisms and, a well-defined reversible voltammetric response with a high heterogeneous ET rate [48, 49]. A wide range of mediating compounds have been identified both inorganic, e.g., potassium ferricyanide [50], and organic, e.g., flavins [51], quinones [52], neutral red [53], and thionine [54]. Depending on whether they are lipid soluble or insoluble they are expected to have different efficiencies [55]. The most investigated mediators in MFCs are phenazines, phenoxazines, phenothiazines, and quinones [38]. However, synthetic mediators often possess instability and toxicity. Additionally, the need of a regular supply with the free diffusing redox shuttles makes the approach technologically unfeasible and environmentally unfriendly. The necessity to develop new innovative configurations of MESs,

where all required active components are securely co-immobilized on an electrode surface, is obvious. Electron conducting redox hydrogels, particularly osmium (Os) RPs, have been successfully used in the development of enzyme-based biofuel cells [56], reagentless biosensors [57] and have received considerable attention towards "wiring" microbial biofilms. The variety of available redox polymers and their applications with respect to various microbial species is discussed in **Paper I**.

1.3. Electron conducting redox polymers

Electron conducting hydrogels are cross-linked polyelectrolytes capable of swelling in water and forming redox hydrogels. These gels conduct ions and enable the diffusion of water-soluble compounds, i.e., reactants and products of a reaction, and have redox features [58, 59]. In this way such hydrogels mediate ET between a biocatalyst and an electrode and, importantly, provide an immobilization matrix for the biocatalyst [60]. Redox hydrogels are comprised of a polymer backbone with redox centers incorporated into the polymer through flexible tethers, which hold them as pendant side groups. Electrons are conducted by self-exchange of electrons/holes in the water-swollen hydrogels from the reduced (electron-loaded) to the oxidized (hole-loaded) mobile redox centers. A schematic representation of the process is depicted in Figure 2. According to the Marcus theory [61] self-exchange redox reactions occur due to collisional ET. The transfer of electrons occurs when the tethered redox centers collide at a distance, which can be crossed over by the electron. This distance limits the rate of the self-exchange process [62]. For this reason, the length of the tethering spacers, which is optimally thought to be 8 to 15 atoms [63-65], greatly affects the MET rates. These flexible structures are able to increase the dislocation amplitude of the redox centers in space and allow effective electron-shuttling collisions [66]. The electron diffusion can also be affected by the applied operating potential. Maximum rate can be obtained at the applied potential of the polymer potential itself in order to keep reduced and oxidized mobile centers at equal density and assure ET resulting exchange between the centers [67].

Redox hydrogels may have diverse chemical structures, e.g., acrylamides, acrylates, methacrylates, linear or branched poly(vinylalcohol)s and poly(ethylenimine)s with incorporated redox centers such as ferrocene [16, 68, 69], cobaltocene [70], quinones [71, 72], phenothiazine [73], and viologen [69] derivative redox cores. The most known and widely used group of redox polymeric mediators is Os-complex modified poly(vinylimidazole) [74] and poly(vinylpyridine) [75] derivatives. These mediators were developed based on the work of Forster and Vos [76] and extensively characterized by Heller and

coworkers and found broad application in enzymatic biofuel cells and biosensors [56, 57]. According to Heller, to achieve a stable electrical wiring of an enzyme with a redox polymer one needs to follow the design principles of a polymeric mediator [59]: (i) good solubility, availability of hydrophobic, charged and hydrogen-binding sites on the polymer to adequately complex an enzyme; (ii) only a small part of the polymeric wire may bind to an electrode surface, the largest fraction should remain unadsorbed to be available to complex with the redox enzyme; (iii) formation of a three-dimensional polymeric network through crosslinking to assure a fast diffusion of electrons, reactant and product molecules of a particular reaction.

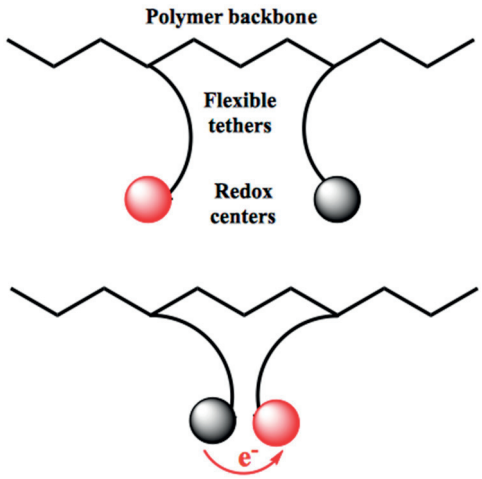


Figure 2. Electron diffusion in a redox hydrogel by collisions between the mobile redox centers.

The broad advanced possibilities in material science allow synthesis of redox polymers with required properties and structures to obtain optimal ET with a biocatalyst. The properties of the OsRPs can be adjusted by varying the structure of the monomer in the composition of the polymeric backbone to moderate hydrophobicity and hydrophilicity, adapting the length of the tethering spacer between the polymeric backbone and the Os redox center, optimizing the redox potential of the OsRP by selecting between a number of ligands associated with the Os core center [77, 78]. The redox potential of a mediator is an essential consideration. It should be adapted to the redox potential of the biocatalyst in order to ensure an optimal potential difference and a sufficient thermodynamic driving force for a fast ET rate [79]. Structural modification of the OsRP with different ligands, electron donating or accepting groups, allows one to choose a more negative or positive potential [80], and provides a wide potential range from

-0.2 V [64, 65] to 0.55 V *vs*. Ag|AgCl [63]. The broad design possibilities for adjustment of the properties of polymeric hydrogels advance their widespread implementation.

Redox polymers have been extensively used and investigated to electrically "wire" redox centers of enzymes to conductive surfaces in various bioelectrochemical applications, e.g., amperometric biosensors, biofuel cells, and biosupercapacitors [60]. The timeline of the employment of RPs in an effort to electronic "wiring" was started in 2004 with the Gram-negative bacterium *Gluconobacter oxydans* mediated by an Os-based RP [81]. All cases of redox polymer-based MET with microbial cells are presented in **Paper I**. The implementation of RP-based mediation in microbial electrochemistry is so far restricted due to the poor knowledge about microbial EET mechanisms, which prevents the development of a rational design of the mediators for that particular purpose.

2. Extracellular electron transfer by Gram-positive bacteria

In 1884 Christian Gram developed a staining method allowing classification of almost all bacteria into two groups based on their staining properties. One group, the Gram-positive bacteria, strongly retains Gram's stain, whereas another one, the Gram-negative bacteria, does not. In 1950s several microbiological, biochemical and microscopic studies provided characteristics demonstrating the difference in composition and architecture of the cell envelope of bacteria with different staining properties [82, 83]. However, a complete understanding of how Gram's procedure differentiates the two groups of organisms was brought about by Terry Beveridge and coworkers. They inspected *Bacillus subtilis* and *Escherichia coli* by transmission electron microscopy and showed how the different structures of the Gram-positive and the Gram-negative bacterial cells affect the staining mechanism [84, 85].

2.1. The cell wall of Gram-positive bacteria

The cell envelope of Gram-positive and Gram-negative bacteria are distinct in several fundamental ways, which strongly influences and largely designates EET capabilities, mechanisms, and pathways of the two kinds of microorganisms. First of all, Gram-positive bacteria lack the outer membrane. Most of the Gram-positive staining bacteria are surrounded by a cell wall, which is many times thicker than that in Gram-negative bacteria [86]. A general scheme of the structure of the cell envelope for a Gram-positive bacterium is presented in Figure 3.

The common feature of all bacterial cell walls is that it is composed of mesh-like peptidoglycan layers. Peptidoglycan is a polymer of repeating two sugar derivatives, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), coupled through β-1,4-linkages (Figure 4A) [87]. The polymer chain length varies considerably among different species [88] from 6-18 disaccharide units for *Staphylococcus aureus* [89] up to 5000 for *B. subtilis* [90]. The polymer chains are

linked together by stem peptides composed of different L-/D-amino acids, which are covalently bound to NAM residues (Figure 4A). A recent nuclear magnetic resonance spectroscopy investigation [91] discovered that each glycan strand forms a right-handed helix, with three NAM-NAG units per turn. This forms a structural design allowing a three-dimensional orientation of the peptides around the axis and a threefold cross-linking for each peptidoglycan strand with three adjacent strands (Figure 4B). Furthermore, in some Gram-positive bacteria the peptidoglycan backbones are additionally cross-linked by interpeptide bridges through covalent connection of the two extended stem peptides (Figure 4A) [86]. The peptide interbridge composition and structure vary considerably from one taxonomic group to another [92-95]. Generally, Gram-positive bacteria have considerably more cross-linking in their peptidoglycan compared to their Gram-negative counterparts [86, 96]. The cell wall in Gram-positive microorganisms form a 30-100 nm thick, rigid but elastic framework with pores ranging from 50 to 500 Å (as exemplified by *S. aureus*) [97], and allowing proteins with a molecular weight up to 50 kDa to pass through [98, 99]. The peptidoglycan plays important roles in determining the shape of the bacterium and stabilizing the cell [100].

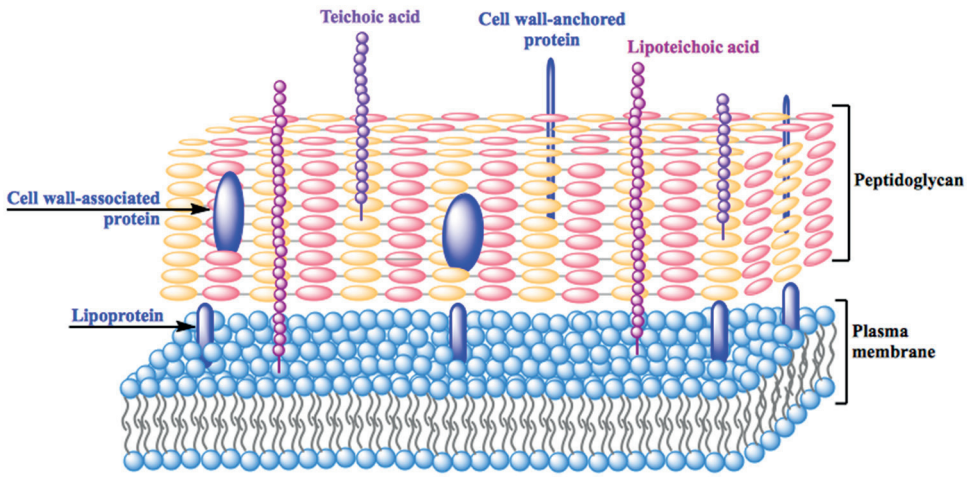


Figure 3. A model of a typical Gram-positive cell envelope.

A distinguishing feature of Gram-positive bacteria is the presence of anionic glycopolymers, which tunnel the peptidoglycan meshwork extending much beyond it [101-103] and constitute up to 60% of the total mass of the cell wall [104-106]. There are two classes of such polymers: One is wall teichoic acids covalently attached to NAM residues of the peptidoglycan [103, 104] and the lipoteichoic acids, which are anchored to plasma membrane lipids [103, 107, 108]. The teichoic acids strongly vary in composition across species [100], but they are

commonly based on glycerol-3-phosphate or ribitol phosphates [109] and have a negative charge by means of their phosphodiester linkages (Figure 5) and contribute to a “continuum of anionic charge” on the cell surface [103, 104]. The anionic continuum is an essential feature of the bacterial cell and allows the binding of extracellular molecules and ions due to electrostatic interaction forces [104]. For instance, it was shown that the teichoic acids are able to bind protons [110] and extracellular metal cations [111, 112]. The principle of interaction with cationic polymeric mediators, described in **Papers II-V**, is based on electrostatic interactions as well. In some Gram-positive bacteria, e.g., *Micrococcus* strains [113], that do not contain wall teichoic acids, other anionic glycopolymers attached to the cell wall perform analogues functions [103].

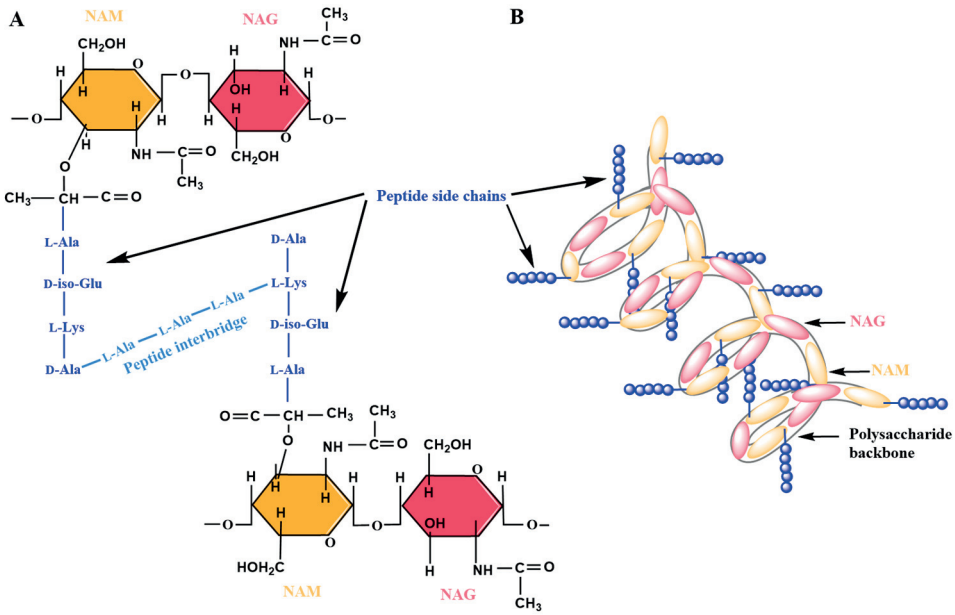


Figure 4. Peptidoglycan structure for *E. faecalis*: (A) Composition of the peptidoglycal with peptide interbridges crosslinking glycan strands. (B) A helical peptidoglycan strand.

The surface charge is dynamic and can be regulated in accordance with the needs of the bacteria and represents adaptation reactions to the environmental conditions. The biosynthesis of the teichoic acids in the cell wall is essentially upregulated when the access of metal ions is limited for the bacteria in the environment, which essentially increases the negative charge [114, 115]. The structure of the teichoic acids can also be adjusted by the attachment of a positively charged D-alanine ester [116] or a variety of oligo- or monosaccharides, commonly glucose or N-acetylglucosamine [103]. The tailoring modification allows neutralizing the

negative charge on the glycopolymer, which hereby reduces the binding capacity of cationic compounds. Such a regulating function of the teichoic acids of the wall plays an essential role in biofilm formation [117, 118], antimicrobial resistance [119, 120], and interactions with various surfaces [103, 104]. Teichoic acids also contribute to a normal cell envelope structure and function; bacterial cells deficient in teichoic acids grow much slower and undergo various morphological anomalies, e.g., increased cell size, non-uniform thickness of the peptidoglycan barrier [121, 122], and even loss of typical cell shape [123-125].

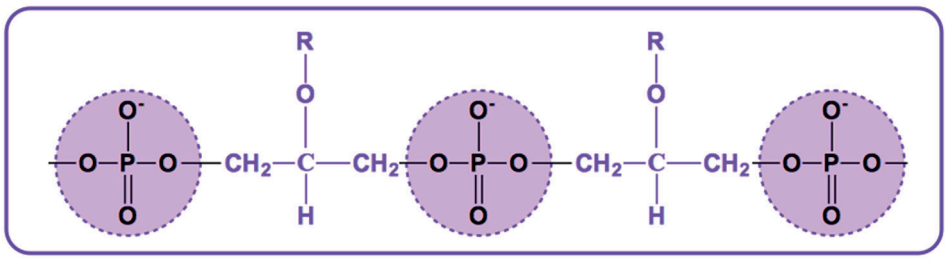


Figure 5. A generalized structure of a teichoic acid represented by glycerol phosphate polymer with a side chain R (R can be D-alanine, glucose or other mono-/oligosaccharides).

Lipoproteins are proteins covalently anchored to the outer side of the cell membrane. They function in the subcellular zone between the cytoplasmic membrane and the peptidoglycan. Additionally, Gram-positive bacteria often have cell wall anchored or associated proteins. Many of them are covalently attached to the peptidoglycan; others are noncovalently bound via ionic interactions to teichoic acids [126-128]. The proteins are involved in peptidoglycan biosynthesis or cleavage, interactions of the bacterial cell with the environment or have a role in virulence, such as adhesion to host tissues [104, 129]. Some highly pathogenic bacterial strains possess capsular polysaccharides, glycopolymers of various structures, which are anchored to peptidoglycan and take part in immune evasion and resistance to phages [130, 131].

2.2. Extracellular electron transfer pathways

Due to the structural characteristics of the cell envelope, Gram-positive staining bacteria were considered as incapable for DET [132]. This group of microorganisms, however, has captured considerable attention in the development of MFC technology, which nowadays represents not only a promising ecologically friendly technology, but also a multidisciplinary research platform for studies of electrical properties and EET mechanisms in microorganisms. Many of the up to

date known electroactive Gram-positive bacteria, which have been attempted to be studied in depth, have been isolated from various MFCs. One of the first Gram-positive bacterium isolated from a mediator-less MFC was anaerobic Fe(III)-reducing *Clostridium butyricum*; its electroactivity was demonstrated using various electrochemical techniques, but no mechanisms or pathways on how electrons enter the metabolism were revealed [133]. More recent work on a *Clostridium*-dominated microbial community has indicated the important role of riboflavin as a mediator in the extracellular metal reduction process of the bacteria [134]. Numerous studies demonstrated the bacteria to be able to produce soluble redox mediators enhancing mineral reduction [13, 42, 135, 136]. Such a self-mediating ET process was shown for an anodic microbial consortium in a mediator-less MFC, which was largely settled with Firmicutes bacteria, such as *Bacillus*, *Clostridium*, *Enterococccus*, *Eubacterium*, *Lactobacillus*, and *Lactococcus* species [9]. Their role was not identified but Rabaey et al. supposed that at least *Clostridium* sp. [133, 137], *Enterococccus gallinarum* [138], *L. lactis* [139], and *Lactobacillus rhamnosus* [139], to be electrochemically active drawing on their previous studies.

L. lactis is closely related to *Enteroccoccus* and can self-catalyze anodic ET by excretion of soluble quinone mediators [45]. The essential role of bacterial quinones in EET has also been shown for Gram-negative bacteria [52, 140, 141] and dealt with in detail for *E. faecalis* in **Papers IV** and **V**. Spore-forming bacteria from the genus *Bacillus* were proven to produce flavins and establish self-mediating ET under anaerobic and aerobic conditions [51, 142]. Extracellular excretion of flavins is a widely known strategy to facilitate ET in the Gram-negative model bacterium *S. oneidensis* [43, 44, 143-146]. The released compounds are redox active and function as electron transfer mediators to link the cells to external minerals [43, 44]. For a number of *Bacillus* species in various microbial consortia it has been generally demonstrated that flavins improve the power generation of MFCs [147-149] in two different ways: (i) by excreting flavins and enhancing MET of other members [147] and (ii) by producing fatty acids and inhibiting the growth of methanogenic bacteria on the anode [149]. Furthermore, a recent study [150] showed that extracellular polymeric substrates surrounding *Bacillus* sp. cells are electrochemically active and play an important transient mediating role in microbial EET. Extracellular polymeric substrates are common compounds for bacteria, which form a three-dimensional structure around the cell and contribute to biofilm formation and protection from undesirable influences from the environment [151-153]. The existence of redox active extracellular polymeric substrates was also confirmed for *S. oneidensis* and the electroactive yeast *Pichia stipites* [150].

Lysinibacillus sphaericus [154, 155] and *Corynebacterium* sp. [156] in mediator-less MFCs have been confirmed to be electroactive sugar-oxidizing Gram-positive

bacteria self-excreting redox compounds. The structures of those compounds remain however, unknown and are yet to be identified.

Certain Gram-positive bacteria undergo DET; this phenomenon is especially of great interest taking into account the structural organization of the cell envelope [157]. The very first reported Gram-positive bacterium capable of direct electrode reduction was thermophilic *Thermincola ferricetica* [158, 159]. Though this Fe(III)-reducing bacterium was thoroughly characterized microscopically, electrochemically and kinetically, the mechanisms of EET is still poorly understood [160, 161]. Another two isolated bacteria, cellulose-oxidizing *S. aureus* [162] and oxygen-reducing *Micrococcus luteus* [163], were demonstrated to be capable of DET, however, no details about the ET processes are yet available. A later study of *Thermincola potens* revealed the involvement of *c*-type cytochromes in the ET across the 37 nm thick bacterial cell envelope during metal ion reduction [164]. Biochemical and biophysical investigations indicated several cell wall-associated multiheme cytochromes *c*:s, being involved in the EET processes performed by this bacterium [20]. Another Gram-positive Fe(III) oxide reducing bacterium, *Carboxydothermus ferrireducens*, was reported to rely on an S-layer of associated *c*-type cytochromes [165, 166]. Cytochromes are essential electron carriers and key players in the EET processes of the model Gram-negative organisms *Geobacter* and *Shewanella*. Outer membrane cytochromes move electrons across the periplasm and the outer membrane [6, 23]. Gram-positive bacteria normally have cytochromes in their respiratory chains, but an abundant presence of multiheme cytochromes is an unusual property [165, 167]. Furthermore, as recent research findings revealed, DET can be carried out by bacterial species lacking cytochromes. As is specifically demonstrated in **Paper IV**, *E. faecalis* is able to move electrons from the DMK pool in the membrane directly to conductive surfaces only when cytochromes are not present in the cell. How the electrons transfer the cell envelope in this case is not clear, but possibly it occurs with the aid of some cell wall/surface-associated proteins. Another gut bacterium, *Listeria monocytogenes,* is known to perform EET; as recently demonstrated this species delivers electrons from membrane-associated DMK to an external electron collector via flavin-based proteins located in the cell wall [21]. Thus, there is no established relationship between the presence of a cytochrome and the ability for DET at least not for Gram-positive bacteria.

2.3. External mediators in extracellular electron transfer

A common way to improve EET of a microorganism is the introduction of various mediators to electrically connect the metabolism to a conductive surface. The use of mediators is especially critical and helpful for Gram-positive bacteria, which, as mentioned above, have a thick non-conducting cell envelope and are often restricted in EET reactions. A number of compounds, artificial as well as naturally existing, have been reported to serve as redox mediators promote electrochemical interaction between various bacteria and electrodes [38, 48].

Flavins are often used as electron shuttles; they constitute prosthetic group of flavoproteins, which are typical membrane-associated electron carriers or enzymes. Flavins may carry two electrons and two protons and, therefore, may serve as external redox mediators for microorganisms. Flavins are synthesized by cells from riboflavin and occur in proteins in the form of flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). For instance, flavin-mediated EET was proven for *Bacillus megaterium* employing a mixture of riboflavin, FMN and FAD [168]. The fermentative bacterium *L. lactis* was also shown to undergo MET using flavins available in the yeast extract medium [169]. However, not all kinds of flavin facilitate EET of this bacterium. Only riboflavin and FMN induced a biocatalytic current, while FAD did not. Such a discriminating functionality of EET by *L. lactis* was explained by the selective binding of riboflavin and FMN to specific sites in the cytoplasmic membrane [170]. Similarly, *E. coli* and *Pseudomonas* sp. produce flavins in comparable quantities to *Shewanella* sp. [44] However, neither *E. coli* nor *Pseudomonas* sp. is able to utilize the secreted riboflavins in the EET as redox shuttles, which can be explained by the existence of a rapid specialized transport of these compounds across the cell membrane [44]. By contrast, the anaerobe *Faecalibacterium prausnitzii* [171] and facultative anaerobe *E. faecalis* [172] display riboflavin-shuttled EET in MFCs.

Quinones constitute another group of electron carriers, however, with a lipophilic nature. Generally, respiring bacteria contain one of the two types of quinones with different potentials: ubiquinones or menaquinones. In this connection *Corynebacterium* sp. [156] and *Desulfitobacterium hafniense* [173] are capable of reducing humic acid anthraquinone-2,6-disulfonate and using it as an electron-carrying mediator in a single-chamber MFCs. Vitamin K_3 efficiently accomplishes MET of *Lactobacillus plantarum* [16]. Menadione-based MET was shown for *E. faecalis* and discussed in **Papers IV** and **V**.

Phenazine compounds produced by *Pseudomonas* species can significantly improve the electricity generation capacity of *Brevibacilli* sp., which is a poor electricity generator when operating alone in MFCs [174]. Artificial mediators

(those are not naturally found in cells), e.g., soluble redox-active cobalt-based complexes, have been applied as mediators to facilitate EET of *Clostridium autoethanogenum* [175]. The shuttles are very stable and approved themself as biocompatible molecules in redox reactions with proteins [176, 177]. Potassium ferricyanide is also used as a mediating compound to carry electrons across the cell envelope in *B. subtilis* [15] and *E. faecalis* (**Paper IV)**. However, it was proved to be non-biocompatible and non-efficient under long-term functioning conditions for *L. plantarum* [16].

From a technological point of view the use of electron mediating RPs, rather than diffusible redox compounds, is more feasible to move MESs toward full-scale applications. Up to now, only three Gram-positive bacterial species have been reported to establish MET using RPs: a number of OsRPs proved to "wire" *B. subtilis* [15] and *E. faecalis* cells (**Papers II-IV**) to electrodes; a ferrocene-based phospholipid polymer successfully mediated *L. plantarum* bacteria [16]; and a quinone-based RP was electronically linked to the metabolism of *E. faecalis* (**Paper V**). Such interactions are complex and need a mechanistic understanding in order to find a general technological application. The features of the electrochemical interaction mechanisms between Gram-positive bacteria and polymeric high molecular weight redox mediators are presented in **Papers III** and **V**.

3. *Enterococcus faecalis*

The term “enterococcus” comes from the end of the 19th century, when in 1899 Thiercelin reported to the French Society of Biology about a saprotrophic diplococcus microorganism inhabiting the digestive tract of humans [178]. He identified the microbe as a Gram-positive bacterium and denominated it “Enterocoque” (from Greek éntero "intestine" and coccos "granule") in order to describe its intestinal origin and coccoid morphology. Thiercelin noticed that the bacterium is pathogenic to mice [178] and is prevalent in the intestinal tracts of persons suffering from diarrhea followed by development of septicemia due to the translocation of the microorganisms from the gastrointestinal tract to the blood stream [179]. The same year, MacCallum and Hastings described a similar organism named *Micrococcus zymogenes* [180], nowadays known as *Enterococcus faecalis*, which caused lethal endocarditis and was reported to be hemolytic. Later in 1906, Andrewes and Horder characterized a microbe from an endocarditis patient, which they called *Streptococcus faecalis* [181]. The organism was given the name to highlight its common pullulation in the intestine of humans. Later, a few species different from *S. faecalis* in terms of fermentation capacities were described, e.g., *Streptococcus faecium* [182] and *Streptococcus durans* [183].

In 1937 Sherman introduced a classification of the streptococci and divided them into pyogenic, viridans, lactic and enterococcus [184]. The enterococci included only a few species, *S. faecalis*, *S. faecalis* var. *liquefaciens*, *S. faecalis* var. *hemolyticus*, *S. faecalis* var. *zymogenes*, and *S. durans*, and were grouped based on the criteria of growing at 10 to 45 °C and at a pH value of 9.6 in 6.5% sodium chloride and survivability after heating up to 60 °C for 30 min. In 1970 Kalina suggested a separation of the enterococcal group into the unique genus Enterococcus due to the phenotypic characteristics of the species [185]. However, this was done only in 1984 based on genetic evidence provided by DNA-DNA and DNA-rRNA hybridization studies [186-188] and 16S rRNA sequence analysis [189]. Currently the genus *Enterococcus* includes 37 accepted species [190] with a wide range of habitats.

3.1. Ecology

Enterococci are Gram-positive non-spore forming bacteria occurring as single cells, pairs or short chains. They are obligatory fermentative chemoorganoheterotrophs, commensal members of the gastrointestinal microbiota in humans and other mammals, birds [191] and insects [192, 193] and some of them are found associated with plants [194]. Living in the nutrient-rich intestinal environments, enterococci are fastidious and require complex nutrients for growth, i.e., some amino acids (valine, leucine, threonine, serine, methionine, arginine, histidine, glutamine) and vitamins (biotin, pantothenate, pyridoxine, riboflavin, folic and nicotinic acids) for growth [195].

When enterococci come out from the intestines of endothermic animals into secondary habitats, i.e., waters, soils, plants, they become stressed by a number of abiotic and biotic factors, which commonly reduce their population over time. Generally enterococci are very hardy and can survive and grow under extreme conditions, i.e., at temperatures between 10 and 45 °C, within a pH range from 4.8 to 9.6 and in the presence of high concentrations of sodium chloride (6.5%) and bile salts (40%). They are also extraordinary resistant to inhospitable conditions such as prolonged desiccation, ionizing radiation, osmotic and oxidative stresses, high concentrations of heavy metals, antibiotics and detergents [190]. Enterococci are capable of an extended survival period, but importantly they do not grow, under nutrient starvation conditions in sterilized sewage and quickly decline in sterilized lake waters and phosphate buffer [196]. Furthermore, it was shown that carbohydrate starvation intensifies resistance to oxidative stress, heating, acids, ethanol and sodium hypochlorite [197-200].

Enterococci are broadly considered as fecal contamination indicators in waters and soils. However, the existence of typically plant inhabiting species has to be taken into consideration [201], since they are not associated with fecal pollution. Most of the Enterococcus genus members are part of commensal microbiota, but some species are opportunistic human pathogens [202], causing a number of diseases e.g., urinary tract infections, endocarditis, bacteremia, neonatal infections, central nervous system infections, abdominal and pelvic infections [203]. This multidrug-resistant group of pathogens is since the 1970s a leading source of hospital-acquired infections [204, 205]. *E. faecalis* and *E. faecium* are important etiological agents of hospital infections [203, 206, 207], in consequence of which they drew considerable attention in medical bacteriology and ecology and have become the most studied *Enterococcus* species. The gained knowledge is exploited in **Papers IV** and **V**.

3.2. Cell envelope

The bacterial cell envelope is a dynamic multilayered protective barrier against the extracellular environment. Most of the obtained knowledge about the cell envelope of enterococci comes from studies on their pathogenesis and antibiotic resistance. The cell wall of *E. faecalis* is about 40 nm thick [208] and, as for all Firmicutes, consists of peptidoglycan, anionic polymers (lipoteichoic and teichoic acids), wall associated and anchored proteins, lipoproteins and polysaccharides. The peptidoglycan multilayers are cross-linked by pentapeptides. L-Ala-D-iso-Glu-L-Lys-D-Ala-D-Ala [209] (Figure 4A). Most of the species in the *Enterococcus* genus form peptide interbridges consisting of a single D-Asp residue [186], *E. faecalis* synthesizes a different crossbridge composition with two or three L-Ala units [87]. The cross-linking is realized in the same way as for all Gram-positive bacteria, via covalent binding of two branching peptide stems in positions 3 and 4 of the amino acids for each side (Figure 4A).

The lipoteichoic acids anchored to the cytoplasmic membrane of *E. faecali*s are composed of glycerol phosphate polymers with attached disaccharide residues of 1,2-linked glucose. The teichoic acids attached to the NAM residues in the peptidoglycan possess a complex structure and contain glucose, galactose, N-acetyl galactosamine, N-acetyl glucosamine and ribitol phosphate [210]. As many other bacteria in order to maintain cationic homeostasis, both the lipoteichoic and the teichoic acids can undergo modification by D-alanylation and glycosylation processes [211].

Enterococci have numerous proteins attached to the cell envelope. For instance, adhesins constitute a widespread and highly conserved cell surface factor in enterococci. They are responsible for adherence to collagen or laminin and important for pathogenesis in animal models [212, 213]. Enterococcal surface proteins were firstly isolated from *E. faecalis* and found to contribute to the virulence in animal models and to biofilm formation [214]. Aggregation substance is a group of surface-bound glycoproteins of *E. faecalis*, encoded on sex pheromone-inducible conjugative plasmids. These proteins mediate donor-acceptor aggregation facilitating plasmid transfer between bacteria [215, 216]. Among cell wall-associated proteins, three different muramidases in *E. faecalis* regulate the cell wall lytic activity and target NAG-NAM residues [217-219]. Lipoproteins constitute a group of highly abundant surface-exposed proteins in the pathogenic *E. faecalis* strain V583 [220, 221] and known to facilitate capture of transported nutrients into the cell [222].

Capsular polysaccharides are glycopolymers linked to the peptidoglycan and found on the external surface of the cell. McBride et al. [223] genetically analyzed more than 100 strains of *E. faecalis* and revealed that the majority of enterococcal

pathogens are equipped with a polysaccharide capsule operon together with multi-virulence and antibiotic resistance features. It was shown that the capsule reduces opsonization and phagocytosis by leukocytes [224].

3.3. Metabolism

Enterococci are lactic acid producing bacteria with a versatile metabolism. All enterococci metabolize at least 15 different carbohydrates and carbohydrate-containing substrates (glucose, lactose, maltose, D-fructose, D-mannose, cellobiose, galactose, ribose, β-gentiobiose N-acetyl glucosamine, amygdalin, arbutin, methyl-β-D-glucopyranoside, salicin, trehalose) [225]. A number of other sugars and sugar alcohols, e.g., adonitol, L-arabinose, D-arabitol, L-arabitol, D-glycodextrin, dulcitol, glycerol, gluconate, inulin, 2-ketogluconate, D-lyxose, mannitol, melizitose, melibiose, α-methyl-D-glucoside, D-raffinose, sorbitol, L-sorbose, and xylitol, can serve as energy source for certain reported species [226]. Furthermore, depending on organism, a variety of other compounds (citric, lactic, malic, α-keto and diamino acids) can be used for energy generation [225]. In spite of the vast number of possible substrates only glucose and to a certain degree mannitol, glycerol and gluconate metabolism have been studied.

Glycolysis in *E. faecalis* is realized via one of the major carbohydrate catabolic pathways: the Embden-Meyerhof-Parnas, the Enther-Doudoroff, and the pentose phosphate pathway. The Embden-Meyerhof-Parnas and the Enther-Doudoroff pathways are very similar and they metabolize hexoses by phosphorylation and further cleavage into triose phosphate intermediates, consequently providing cells with ATP by means of substrate phosphorylation. The pentose phosphate pathway catabolizes hexoses, pentoses and sugar acids, producing NADPH and several precursor metabolites for nucleotide biosynthesis [227]. At the expense of glucose, *E. faecalis* exclusively uses the Embden-Meyerhof-Parnas pathway [228-230].

Pyruvate, the final product of glycolysis, is metabolized further depending on the environmental conditions. Enterococci lack the tricarboxylic acid cycle and typical electron transport chains and produce most of its ATP via glycolytic substrate level phosphorylation and regenerate NAD^+ through pyruvate reduction primary to lactate. Although enterococci are homofermentative, some amount of acetate, ethanol and formate are always present; their proportion rises under more alkaline pH conditions [231]. When the bacteria switch from anaerobic to aerobic conditions the metabolism changes from lactate toward acetate and CO_2 [232].

3.4. Respiration

A few enterococcal species are known to be able to respire under certain conditions. *E. faecalis* can synthesize an electron transport chain and drive oxidative phosphorylation under aerobic conditions. Enterocooci cannot synthesize heme [233]. However, certain species of lactic acid bacteria form one or more heme proteins if the cells are supplied with heme [234, 235]. Under such conditions *E. faecalis* assembles two heme-containing proteins, a membrane-bound cytochrome *bd* oxidase [236, 237] and a cytoplasmic catalase [238]. Consequently a simple respiration chain, consisting of NADH dehydrogenase, a demethylmenaquinone (DMK) pool and cytochrome *bd* oxidase can be formed (Figure 6).

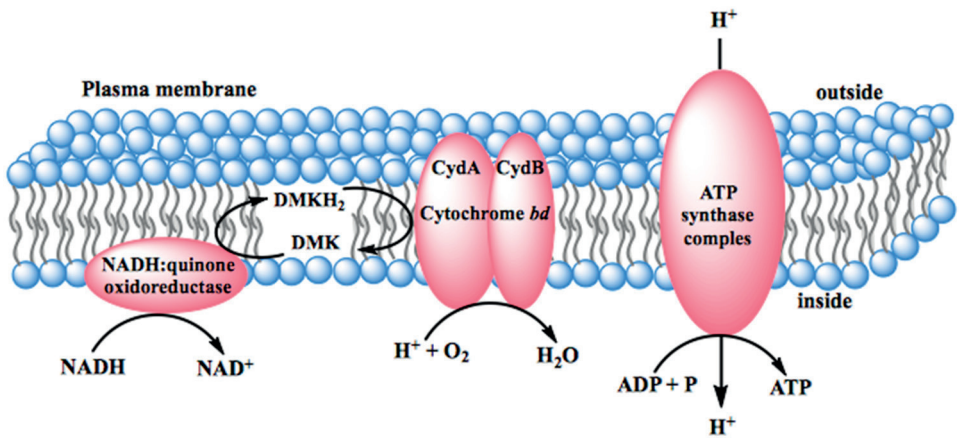


Figure 6. Scheme of the aerobic respiratory chain formed in the *E. faecalis* cytoplasmic membrane when the cells are supplied with heme.

Cytochrome oxidases of *bd*-type are found exclusively in prokaryotes, both in Bacteria and Archaea. The enzyme consists of two different protein subunits, CydB (≈ 36-43 kDa) and CydA (≈ 52-58 kDa), containing three heme prosthetic groups, heme b_{558}, heme b_{595}, and heme *d* [239, 240]. Cytochrome *bd* is a key respiratory protein for *E. faecalis.* It couples oxidation of quinols and reduction of molecular oxygen to water with the formation of an electrochemical gradient across the cytoplasmic membrane.

Quinones are essential electron transport carriers associated with membranes. In general, bacterial quinones are derivatives of ubiquinones or menaquinones. *E. faecalis* contains 2-solanesyl-1,4-naphthoquinone, termed demethylmenaquinone (DMK) (Figure 6) [241]. Quinones are synthetized under both anaerobic and

aerobic conditions [242, 243]. Lactic acid bacteria can also take up quinones or precursors from the environment [242, 244, 245]. DMK can be reduced by various membrane-associated dehydrogenases, including a type II NADH:quinone oxidoreductase. Consequently, when *E. faecalis* cells are supplemented with heme and molecular oxygen, they respire by the oxidation of NADH generated in glycolysis and by the activity of pyruvate dehydrogenase. This allows a higher growth and ATP synthesis yield under aerobic conditions [242, 246, 247]. Aspects of EET by *E. faecalis* cells with and without a respiratory system are extensively discussed in **Paper IV**.

4. Electrochemical methods

4.1 Basic electrochemical principles

A variety of electrochemical methods are focused on processes and factors affecting the charge transport across the interface between different chemical phases. When an electrode, i.e., an electronic conductor, is submerged into an electrolyte, i.e., an ionic conductor, both phases having mobile charge carriers, there will always appear at the interface between the two an electrical double layer (EDL) as a result of a spontaneous reorientation of the mobile charge carriers leading to a charge separation between the two phases which is equal to a potential drop across the boundary.

The structure of the EDL can be described using the simplified model proposed in 1947 by D. C. Grahame [248]. The EDL consists of the Stern layer (also known as the Helmholtz, inner or compact layer) and the diffuse layer (sometimes defined as the Gouy layer) (Figure 7). The Stern layer comprises the inner Helmholtz plane, which is composed of a strongly bound monolayer of oriented solvent molecules in combination with specifically adsorbed ions or molecules, and the outer Helmholtz plane, which is formed by non-specifically solvated adsorbed ions at their closest distance to the electrode surface and to a varying extent oriented solvent molecules. The intermediate three-dimensional region between the compact layer and the bulk solution consists of solvated counter-ions. The total thickness of the EDL does not exceed 10 nm in water-based solutions with an electrolyte concentrations >10 mM.

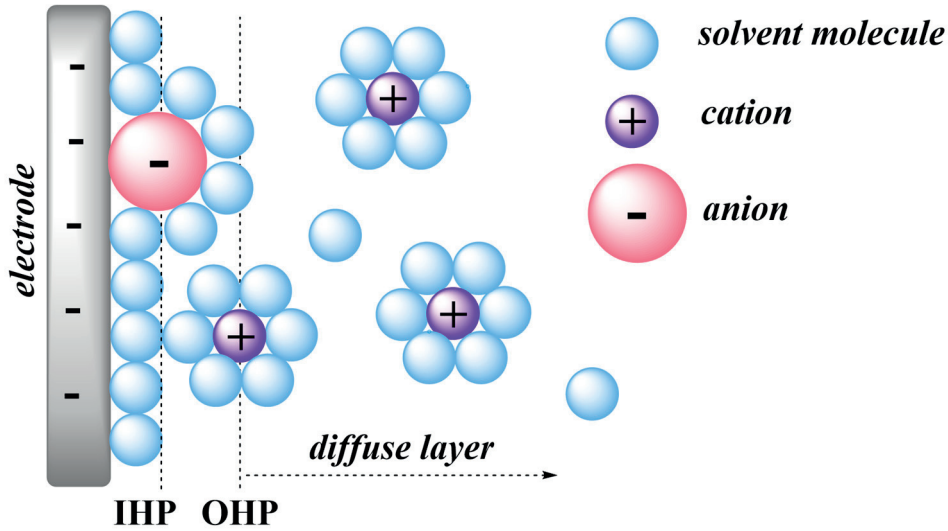


Figure 7. Schematic representation of the electrical double layer structure. IHP – inner Helmholtz plane, OHP – outer Helmholtz plane.

When the potential drop between the two phases, usually expressed as the electrode potential, changes, according to the electroneutrality principle, the excess of charge should be compensated by a redistribution of oppositely charged ions from the solution, that results in a non-faradaic capacitive current I_c:

$$I_c = \frac{\Delta E}{R_s} e^{-t/(R_s C_{dl})} \tag{1}$$

$$I_c = \nu \cdot C_{dl} \tag{2}$$

where R_s is the solution resistance, C_{dl} is the EDL capacitance, t is time, ΔE is the potential change, ν is the potential scan rate in linear sweep electrochemical techniques (*vide infra*).

Since the non-faradaic I_c may add a significant input to the registered total current output, especially in case of porous electrodes with a highly developed surface area, the capacitive current should be taken into account when planning experiments and carefully extracted from the obtained data for a proper evaluation of the (bio)electrocatalytic performance of the system.

Processes involving charge transfer across the electrode/electrolyte interface are called faradaic. A simple ET reaction can be described by the equation:

$$Ox + ne^- \rightleftarrows Red \tag{3}$$

where *Ox* and *Red* are the oxidized and reduced species, respectively, *n* is the number of electrons transferred.

The equilibrium redox potential (*E*) for the electrode in this case is governed by the thermodynamic Nernst equation:

$$E = E^{0\prime} + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]} \tag{4}$$

where $E^{0\prime}$ is the formal electrode potential, *R* is the gas constant (~8.314 J mol^{-1} K^{-1}), *T* is the absolute temperature in K, and *F* is the Faraday constant (~9.6485 $\cdot 10^4$ C mol^{-1}).

When the potential is changed from its equilibrium state, i.e., when a certain overpotential, η, is applied, the surface concentrations of *Ox* and *Red* should be reestablished in accordance with the values demanded by the Nernst equation. This requires a current to flow through the interface electrode/electrolyte: an anodic current in the case of a positive shift in electrode potential and a cathodic current as a result of a negative potential change. The relationship between the current (*I*) and η can be described by the Butler-Volmer formalism for a one-ET process:

$$I = FAk^0 \left[C_{Ox}(0,t) e^{-\alpha F\eta/RT} - C_{Red}(0,t) e^{(1-\alpha)F\eta/RT} \right] \tag{5}$$

where *A* is the electrode area, $C_{Ox}(0,t)$ and $C_{Red}(0,t)$ are the concentrations of *Ox* and *Red* at the electrode surface (t =0), α is the dimensionless ET coefficient (takes values between 0 and 1), k^0 is the heterogeneous standard rate constant representing the kinetic facility of the redox reaction [249].

For experimental evaluation, the applied form of Equation (5) is commonly used:

$$I = AI_0 \left[e^{-\alpha nF\eta/RT} - e^{(1-\alpha)nF\eta/RT} \right] \tag{5a}$$

where I_0 is the exchange current, representing conditions at η = 0, when the rates of the oxidation and the reduction processes are equal.

If k^0 is large enough so that the Nernstian relationship remains valid at any η, the electrochemical system is defined as reversible and is controlled only by mass transfer limitations, whereas systems displaying k^0 < 0.005 cm s^{-1} are termed as irreversible and are limited by the ET rate. The term irreversible means here that when sufficiently large a η value is applied for a noticeable current to flow, it only flows in one direction. Transitional type is *quasireversible* electrochemical systems, where a certain overpotential is required to drive a current flow through the electrode/solution interface and both anodic and cathodic processes have a

significant impact on the current output in the overpotential region, where mass-transfer limitations are not substantial [249].

One should not mix up the electrochemical reversibility described above and chemical reversibility of the reaction, which means reverse of the reaction when the current direction is changed.

When the experiment is carried out at long timescales or the reaction rate is significant, mass transport of the reactants becomes the limiting factor determining the observed current output. Diffusion limitations may be overcome by using rotating electrodes, solution stirring, or placement in a flow system.

4.2. Experimental electrochemical techniques

A typical three-electrode setup for electrochemical experiments consists of a working (WE), which is the object of interest, a reference (RE) and a counter (CE) electrode separated by the electrolyte phase and connected to the measuring unit, a potentiostat, This allows performing controlled perturbation of the WE and detection of the targeted signal (Figure 8). The WEs in **Papers II-V** were made of graphite rods modified with the bacterium *E. faecalis*, and the bacterial cells were immobilized either directly in the absence or the presence of an intermediate layer of a redox mediator on the electrode surface.

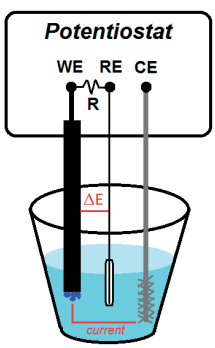


Figure 8. Schematic representation of an electrochemical setup employing a three-electrode system.

Employment of a RE is necessary to set the potential of the WE as a known difference value of the potential between the WE and the RE, the potential of the later should be stable and constant within the whole range of experimental conditions. The RE is connected to the potentiostat through a high input resistance, and therefore, only a very negligibly small current flows though the RE with no influence on its potential value. To unify the results obtained using different REs,

the potential of the WE is typically referred to the potential of the standard hydrogen electrode (SHE) declared as the zero potential. An Ag|AgCl|KCl_{sat} (+0.199 V vs SHE) and an Ag|AgCl|$KCl_{0.1\ M}$ (+0.288 V vs SHE) were used as REs in the works presented in this thesis.

The purpose of the CE (also denoted the auxillary electrode) is to supply the current required to the reactions occurring at the WE without any limitations of the observed response. Therefore the CE should be made of an electrochemically inactive material with a significantly higher surface area compared to that of the WE. A platinum mesh or platinum wire was employed as the CEs in **Papers II-V**.

The various electrochemical methods can be divided into two main groups. The dynamic methods are based on the observation of the current response, when the system is disturbed from its equilibrium state by applying a controlled external excitation, e.g., current or potential. When static techniques are involved, no current flows across the interface between the electrode surface and the electrolyte. E.g., open circuit potentiometry (OCP) is based on monitoring the WE potential over time. Cyclic voltammetry, amperometry and electrochemical impedance spectroscopy (EIS) are dynamic methods and were used in **Papers II-V**.

4.2.1. Cyclic voltammetry

In cyclic voltammetry the potential applied to the WE is linearly swept over time with a constant scan rate from the initial potential E_1 until a switching potential E_2 is reached (Figure 9A). At this point the potential sweep direction is reversed either to the initial potential E_1 (finishing the first cycle) or further to the potential E_3. In the latter case the potential window for the cyclic voltammograms (CVs) ranges within E_2 and E_3, this is mainly related to the experiments, when cyclic voltammetry is preceded by OCP, and the E_1 value corresponds to the OCP of the system. The potential scan rate for the experiment may vary significantly, depending on the reaction rate, the properties of the WE and target parameters. Typically in bioelectrochemistry, the CVs are obtained within the range of v from 0.1 mV s^{-1} (under slow electrochemical kinetics or/and high electrode capacitance) to 100 mV s^{-1}.

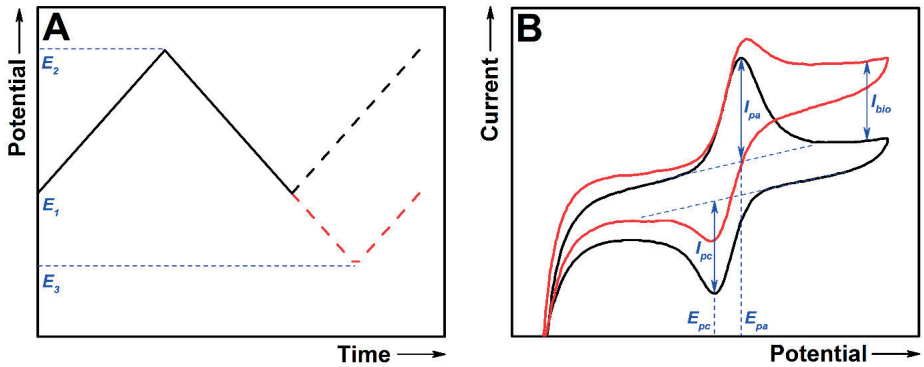


Figure 9. (A) Potential-time profiles for cyclic voltammetry. (B) Typical CVs for a bioelectrode based on MET between the bioelement and the electrode surface in the absence (black curve) and in the presence of substrate (red curve).

Cyclic voltammetry was employed in **Papers II-V** to evaluate the bioelectrocatalytic current (I_{bio}) generated by the *E. faecalis* cells and to electrochemically characterize a number of OsRPs in **Paper III**. I_{bio} was calculated as the difference between the background current registered in the absence of substrate and when glucose was provided to the bacterial cells as a source of electrons for the respiration chain (Figure 9B), which was coupled to the electrode reactions.

Since relatively thick films of RPs were employed in **Papers II-V** to mediate the ET between *E. faecalis* cells and the electrode, the electrochemical behavior of such a redox system is limited by the rate of electron hopping through the RP matrix, which obeys the law of diffusion. The peak current (I_p) for an electrochemically reversible reaction under diffusion control can be calculated using Randles-Ševčík equation [250], Equation (6):

$$I_p = 0.4463\, nFAC\left(\frac{nF\nu D}{RT}\right)^{1/2} \qquad (6)$$

where C is the bulk concentration of redox active compound, D is the diffusion coefficient. At 25 °C Equation (6) takes the form of Equation (6a):

$$I_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C \upsilon^{1/2} \qquad (6a)$$

For the RP films C can be replaces by Γ/l, where Γ is the projected surface coverage, l – the thickness of the film.

Since the Γ value is directly proportional to the amount of charge (Q) necessary to fully reduce or oxidise the immobilized redox active compounds:

$$\Gamma = \frac{Q}{nFA} = \frac{\int IdE}{\nu} \cdot \frac{1}{nFA} \tag{7}$$

The $E^{0\prime}$ value can be determined as the mean value between the peak potentials corresponding to the anodic and cathodic peak currents (E_{pa} and E_{pc} in Figure 10B, respectively):

$$E^{0\prime} = \frac{E_{pa} + E_{pc}}{2} \tag{8}$$

To investigate the kinetics of the ET processes in the system, where diffusion-controlled processes play a significant role in the ET, the method proposed by R. S. Nicholson for quasireversible reactions can be used [251]. Calculation of k^0 using this approach is based on employment of a dimensionless constant, ψ (for the cathodic process):

$$\psi = \frac{(D_{Ox}/D_{Red})^{\alpha/2} k^0}{(\pi D_{Ox} f \nu)^{1/2}} \tag{9}$$

where D_{Ox} and D_{Red} correspond to the diffusion coefficients for the oxidised and reduced species, respectively, and $f = nF/RT$ [249]. Commonly for the redox polymers it can be assumed that $D_{Ox} = D_{Red} = D$, and therefore

$$k^0 = (\pi D_{Ox} f \nu)^{1/2} \psi \tag{9a}$$

If the thickness of the redox layer is low enough to avoid any diffusional limitations or a monolayer of the redox polymer is applied, the *I-E* behavior can be described by Equation (10) [252]:

$$I = \frac{n^2F^2}{RT} \frac{\nu A\Gamma \left(\frac{[Ox]}{[Red]}\right) \exp\left\{\left(\frac{nF}{RT}\right)\left(E - E^{0\prime}\right)\right\}}{\left[1 + \left(\frac{[Ox]}{[Red]}\right) \exp\left\{\left(\frac{nF}{RT}\right)\left(E - E^{0\prime}\right)\right\}\right]^2} \tag{10}$$

and the peak current can be defined as

$$I_p = \frac{n^2F^2}{4RT} \nu A\Gamma \tag{11}$$

In case of a reversible reaction the kinetic parameters of the immobilized redox species in the absence of diffusion limitations can be evaluated by analysis of the

relationship between E_p and $log\ v$ at scan rates, when the difference between $E^{0\prime}$and E_p is greater than 0.1 V, as proposed by E. Laviron [253, 254].

Cyclic voltammetry is one of the most popular methods in microbial bioelectrochemistry. The interested reader may be referred to the review by Patil et al. [47] and related work for a deeper investigation of this subject [255].

4.2.2. Amperometry

In this thesis amperometric techniques were employed for the long-term monitoring of the generated current response under a constant applied potential. Contrary to CVs, amperograms do not contain the input from a non-faradaic current, if a sufficient equilibration time was applied, and allow a direct evaluation of the (bio)electrocatalytic current response.

Amperometric experiments were performed in a standard electrochemical cell with a motionless electrolyte (Figure 9) or using a flow injection analysis (FIA) system connected to a three-electrode flow-through electrochemical wall jet cell (Figure 10). The FIA design allows a controllable laminar flow rate and introduction of a targeted concentration of substrate with a predefined dilution factor. The wall jet cell consists of two compartments, which can be screwed to optimize the cell volume and the distance from the inlet of the solution to the WE to achieve a perpendicular flow to the WE surface. The CE in the form of a platinum ring was mounted in the cell encirculating the WE, whereas the Ag|AgCl|$KCl_{0.1\ M}$ RE was connected to the working volume through an additional chamber in the back wall of the cell.

A typical amperogram obtained using the FIA system is a set of separate sharp peaks corresponding to different concentrations of the substrate injected. The maximum steady state current for each concentration is used as a characteristic parameter.

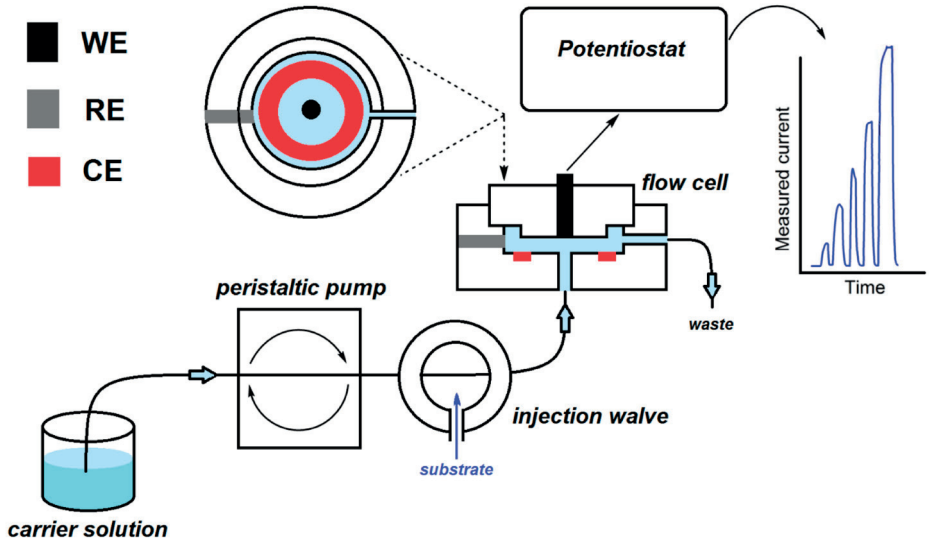


Figure 10. Schematic representation of the electrochemical setup involving a three-electrode system for experiments in flowing electrolyte solutions. Sectional top and side views of the flow cell are presented.

Transport-limited current (I_{lim}) in the wall jet cell with an inlet diameter smaller than the diameter of the WE can be described by Equation (12) based on the analytical theory developed by W.J. Albery and C.M.A. Brett [256-258]:

$$I_{lim} = 1.59 k_c n F C D^{2/3} \nu_c^{-5/12} a^{-1/2} r^{3/4} V_f^{3/4} \quad (12)$$

where k_c is the experimentally determined cell constant, which is close to 0.9 in the original works and in early work by Yamada and Matsuda [259], where a similar equation has been proposed experimentally, V_f is the volume flow rate, a is the jet diameter, r is the radius of WE, ν_c is the kinematic viscosity of the solution.

4.2.3. Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is a non-destructive and a semi-quantitative technique, which can bring information about the electrochemical behavior of bound or mobile charges in a wide range of reaction rates. EIS experiments can be used for simultaneous determination of interfacial parameters, e.g., rate constants, capacitance values, diffusion coefficients, and material features, viz. conductivity, film thicknesses or mobility of bulk charges [260].

In EIS experiment a sinusoidal potential E_t with a small single-frequency amplitude perturbation (normally <10 mV) is superimposed upon the applied

potential and the current response I_t is analyzed [261, 262]. A small amplitude perturbation is necessary to keep a linear relationship between E_t and I_t:

$$E_t = \Delta E \cdot \sin 2\pi f t \tag{13}$$

$$I_t = \Delta I \cdot \sin(2\pi f t + \varphi) \tag{14}$$

where ΔE and ΔI are the maximum amplitude of the applied potential and the current response, respectively, f is the frequency of the potential perturbation, t is time, and φ is the phase angle between the potential amplitude perturbation and the current response.

Equation (15) takes into account that the impedance (Z_t) is analogous to a generalized resistance,

$$Z_t = Z_0 \cdot \frac{\sin(2\pi f t)}{\sin(2\pi f t + \varphi)} \tag{15}$$

where Z_0 is a magnitude.

Applying Euler´s formula, impedance can be represented as a complex function involving an imaginary unit (j):

$$Z_t = Z_0 \cdot (\cos\varphi + j \cdot \sin\varphi) \tag{16}$$

which is composed of a real part $Z' = Z_0 \cdot \cos\varphi$ and an imaginary part $Z'' = Z_0 \cdot j \sin\varphi$.

EIS data can be presented as a Nyquist plot (Arland diagram, where the real and the imaginary parts of the impedance are plotted on the X- and Y-axis, respectively) and a Bode plot, representing the dependence of the impedance or the phase angle on frequency (usually on *log f*) (Figure 11). Similarly to the Bode plot, each point on the Nyquist plot corresponds to the impedance at one frequency.

The common way of analyzing EIS data is fitting the obtained experimental data to a chosen equivalent circuit model based on the physicochemical parameters of the system. It can consist of circuit elements combined in series or in parallel: resistors (R), capacitors (C), Warburg impedance (Z_W) representing mass transfer resistance or pseudocapacitance associated with ET, inductance (L) and a variety of advanced elements intended to mimic the particular experimental conditions and the electrochemical parameters.

Fitting can be performed using dedicated software, e.g., Elchemea Analytical from S. Koch, C. Graves and K. V. Hansen (DTU Energy), Echem Analyst from Gamry

Instrument Inc., ZView from Scribner Associates Inc. or ZSimpWin from Princeton Applied Research used in **Papers III, IV and V**.

A simple ET reaction at the electrolyte/electrode interface can be described by the Randles equivalent circuit, proposed by J.E.B. Randles in 1947 [263] and consisting of an active (uncompensated) solution resistance (R_s) in series with a parallel combination of the capacitor representing the double layer capacitance (C_{dl}) with the faradaic impedance, which has the form of the charge transfer resistance (R_{ct}) connected in series with the Warburg impedance.

The low frequency range (<1 Hz) of the Randles circuit corresponds to a faradaic impedance of the system, since at $f \rightarrow 0$ $Z_C = 1/(j \cdot C \cdot 2\pi f) \rightarrow \infty$ and can thus be neglected, whereas the increase in the frequency leads to a more significant input from the capacitive part. At $f \rightarrow \infty$ the Randles plot contracts into the intercept on the X-axis, corresponding to the R_s value [262].

The electrical double layer at the electrode-electrolyte interface rarely possesses an ideal pure capacitive behavior. To reflect on this deviation, a constant phase element (CPE) is typically introduced into the equivalent circuit.

$$Z_{CPE} = 1/[(j \cdot 2\pi f)^{\alpha} Q] \tag{17}$$

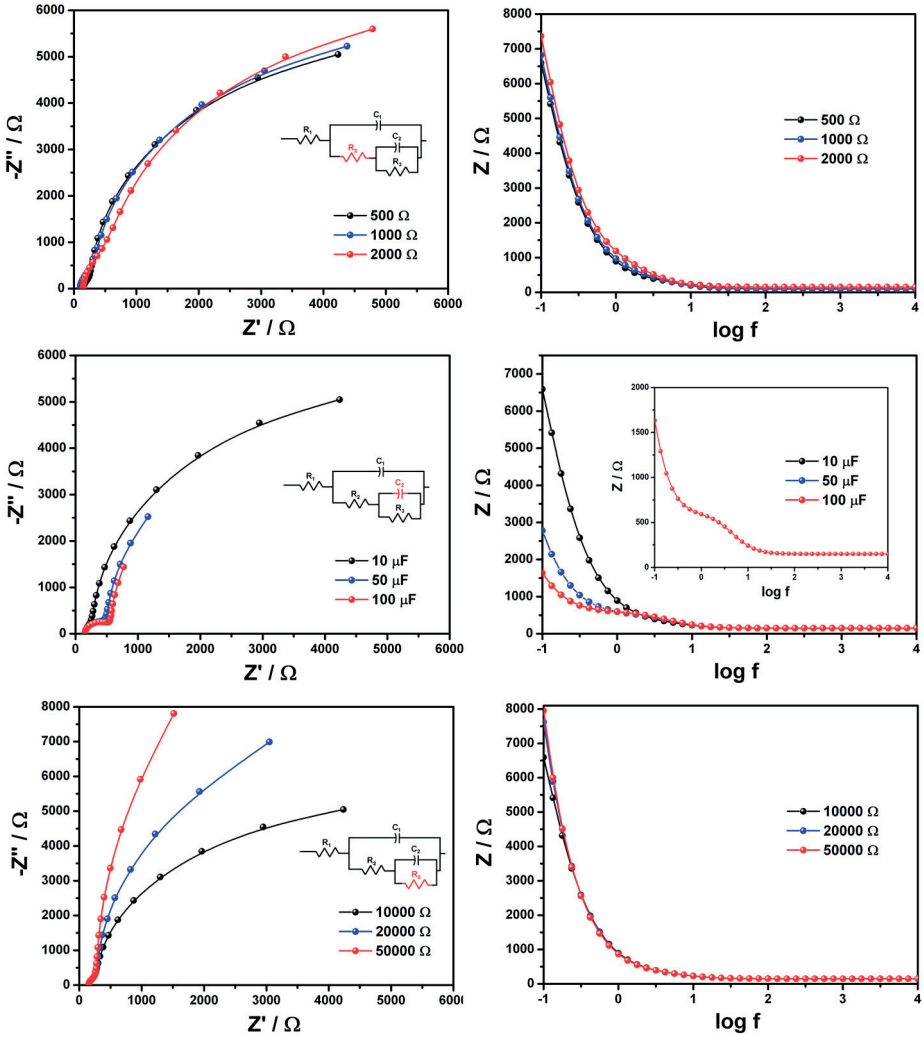
where Q is a CPE parameter, which has dimensions S s^{α} and corresponds to C if $\alpha = 1$. When α is equal to 0.5, the CPE is analogous to the Warburg element representing an infinite length diffusion. The range $0.9 < \alpha < 1$ is attributed to the capacitance of a rough or a porous electrode, whereas $\alpha < 0.9$ reflects the input from the insulating components at the surface.

The effective capacitance, C_{CPE}, can be extracted from the parameters of the CPE for the Randles circuit using Equation (18):

$$C_{CPE} = [Q(R_s^{-1} + R_{ct}^{-1})^{\alpha-1}]^{1/\alpha} \tag{18}$$

Equivalent circuits in multicomponent systems take a more complex form to reflect the separation of charges and characterize the electrochemical behavior in detail. Some examples for electrodes with deposited bacterial cells are summarized in Table 1.

It should mentioned, however, that interpretation of the multivariable EIS spectra may be embarrassed, since several different models have identical impedance *versus* frequency response characteristics with reasonable goodness of fitting, but the physical meaning of related components can be meaningless. Therefore, the results of EIS analysis should be confirmed by other electrochemical techniques, viz. amperometry, voltammetry, etc., or by simulation using developed physical models aimed to describe the phenomena of interest [264].


-Z'' / Ω
Z' / Ω
500 Ω
1000 Ω
2000 Ω
Z / Ω
log f
10 μF
50 μF
100 μF
10000 Ω
20000 Ω
50000 Ω

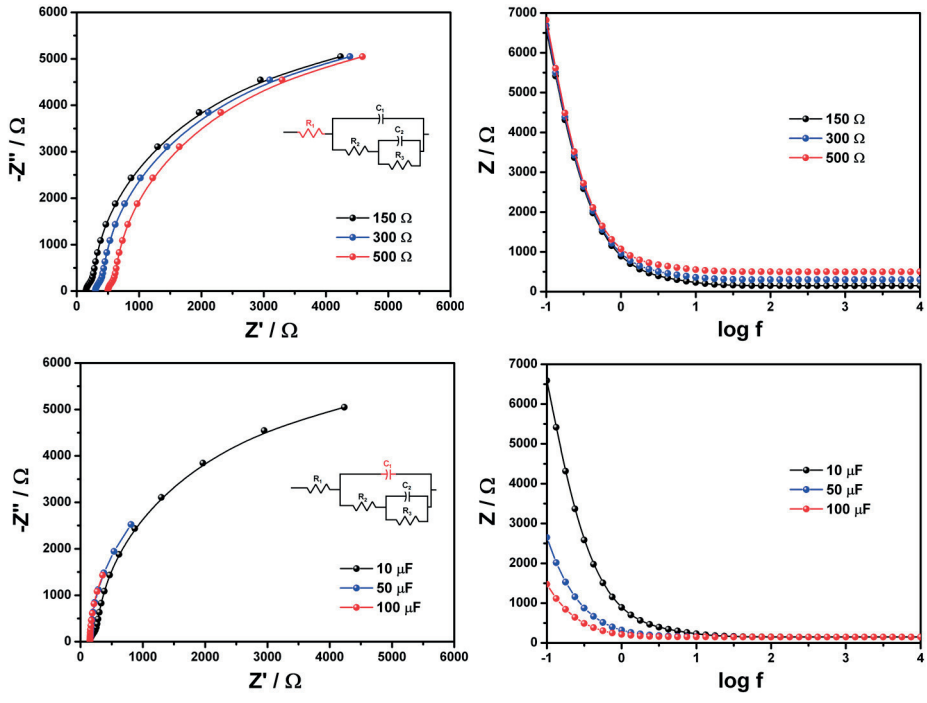
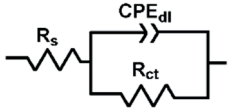
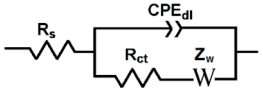
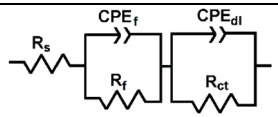
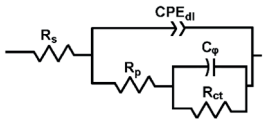
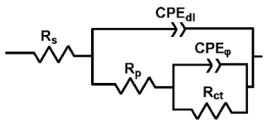
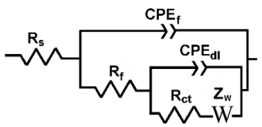


Figure 11. Influence of the equivalent circuit parameters on the shape of Nyquist plots (left) and Bode plots (right). The circuit $R_1(C_1(R_2(C_2R_3))$ used in modified versions in **Papers III-V** with the initial parameters R_1 = 150 Ω, C_1 = 100 µF, R_2 = 500 Ω, C_2 = 100 µF, R_3 = 10000 Ω was applied as a basic spectrum (black curves).

Table 1. Representative examples of the employment of various equivalent circuits for characterization of the electrochemical communication between bacterial cells and the electrode surface.

Equivalent circuit	Elements	Examples	References
	R_s – solution resistance; CPE_{dl} – CPE corresponding to the electrical double layer; R_{ct} - charge transfer resistance	*Geobacter sulfurreducens* on graphite paper	[265]
		Complex anodic biofilm on graphite felt	[266]
	Z_W – open Warburg element	Mixed anodic culture on microfibrous carbon paper	[267]
		Mixed anodic culture on carbon felt	[268]
		P. aeruginosa in mixed culture on carbon ink	[269]
		Salmonella typhimurium suspension on interdigitated microelectrodes	[270]
		S. oneidensis on indium tin oxide	[271]
		S. oneidensis on gold-sputtered carbon paper	[272]
	CPE_f – CPE associated to a bacterial film, R_f – film resistance	Mixed cathodic culture containing *Dehalococcoides* spp. on carbon paper	[273]
	R_p – polarization resistance, combining pore resistance, internal resistance of the mediator (coating) and effect from the concentration polarization of mediator; CPE_φ or C_φ - elements representing pseudocapacitance of the redox polymers	*Bacillus* sp. on copper coatings	[274]
		Streptococcus mutans on metal dental alloys	[275]
		Sulfobacillus thermosulfidooxidans on carbon paste electrodes	[276]
		*E. faecalis* cells on graphite surface modified with Os and quinone-based RPs	**Paper III-V**
		Complex anodic biofilm on carbon paper	[277]
		Mixed anodic culture on fluorinated tin oxide surface. Capacitance elements were used instead of CPEs, film capacitance and double layer capacitance elements were interchanged.	[278]

Summary of publications and future perspectives

Microorganisms, which undergo EET processes, are the principal players of various biotechnological applications, e.g., MFCs and microbial electrosynthesis, and key contributors to global energy and material cycles. The effective electrochemical interaction between living cells and electrodes is a complex event, which leads to an improved performance of microbial electrochemical devices and their implementation into real-life applications. **Paper I** gives a short critical overview of the current situation in the field of microbial electrochemistry, discussing means and possible ways to an optimized and effective electrochemical communication of microbial cells with conductive surfaces.

Most of the microbes attempted to be investigated have been Gram-negative bacteria, potentially strong electricity producers. In contrast, Gram-positive bacteria are thought to be weak electricigens, since they have a thick non-electron conductive envelope preventing EET. Although the mechanisms of electroactivity for Gram-positive staining bacteria are poorly understood, the bacteria (including Enterococci) are usual members of MFCs consortia and potentially can improve the performance. OsRPs are electron shuttles drawing considerable attention because of their ability to efficiently mediate interfacing ET between a biocatalyst and an electrode surface and serve as an immobilization matrix for microbial cells. They are especially attractive in MFCs due to the sustainability of their use. In **Paper II** *E. faecalis* was tested using amperometry and cyclic voltammetry and was shown to transfer electrons generated in glucose metabolism to electrodes via OsRP matrix. *E. faecalis* is the second Gram-positive bacterium (after *B. subtilis*) capable of establishment of an effective electronic communication with an electrode surface mediated by OsRP.

Though the electrochemical communication is well established, the basic mechanisms of electronic interactions between the high molecular weight polymeric mediator and Gram-positive bacteria carrying a thick envelope are not clear. In **Paper III** different OsRPs of various redox potentials and chemical structures were employed to electrochemically characterize the mediated *E. faecalis*-electrode ET interaction using amperometry, EIS and cyclic voltammetry. The obtained results of the characterization suggested a possible penetration of the

RPs into the bacterial cell envelope in order to reach the cell membrane and pick up electrons.

However, the fact that *E. faecalis* is able to transfer electrons with the aid of OsRPs does not add any essential information about the EET mechanism itself. Microbial EET mechanisms and pathways are critical factors in the development and optimization of MESs. To date though many microbial species are identified to be electroactive and able to transfer electrons between the inside and the outside of the cell membrane, the molecular aspects of the microbial ET are poorly understood and have been intensively investigated in the limited model bacterial species, *Shewanella* sp. and *Geobacter* sp. *E. faecalis* is a Gram-positive non-spore forming bacteria, obligatory fermentative chemoorganoheterotroph and a commensal member of the gastrointestinal microbiota in humans and other mammals. The enterocooci fail to synthesize protoporphyrins, the heme metabolic precursor, and for this reason it cannot naturally synthesize cytochromes. However, *E. faecalis* is able to form several heme proteins if supplied with heme or its analogues externally. Under such conditions it assembles two heme-containing proteins, a cytoplasm located catalase and a membrane-embedded bound cytochrome *bd* oxidase. Consequently, under aerobic conditions, a simple respiration chain, consisting of NADH dehydrogenases, a DMK pool in the membrane and membrane-bound cytochrome *bd* oxidase can be formed. In **Paper IV** the presence of cytochromes in the cells was controlled by varying the amount of heme available during the growing step. Using various mediating structures and *E. faecalis* wild-type strain and its mutant line with mutations within the respiratory chain, the bacterium was demonstrated to transfer electron via the DMK pool and not through the membrane-bound cytochromes, which are the key functioning components of EET in many cases for a number of Gram-negative bacteria. The negative effect of the involved cytochrome *bd* oxidase on the EET was demonstrated using amperometry and EIS. Additionally DET has been registered for only cells having DMK in the membrane. However, the obvious question is how it can be realized in *E. faecalis* having a thick cell envelope. This is not clear so far. The findings provide mechanistic knowledge about EET, which is of high interest from a fundamental point of view and for biotechnological applications, e.g., MFCs. Furthermore, *E. faecalis* is an opportunistic human pathogen and its EET pathway can be a possible mode of electrical cell-to-cell connection with other microbes in the gut microflora.

As demonstrated in **Papers II-IV**, *E. faecalis* is able to efficiently transfer electrons via an OsRP matrix, however, the lack of mechanistic knowledge of how such ET communications happen does not allow a rational design development of mediators and their further employment into microbial electrochemical applications. The availability of the mutant line and findings revealed in **Paper IV** enabled a detailed investigation of the ET interaction between *E. faecalis* bacterial

cells and various quinoid mediators using EIS, amperometry, and cyclic voltammetry. In order to control the DMK activity in the cells, quinoid mediators, both polymeric and monomeric, were tested with the wild-type and mutant type devoid of quinones in the cell membrane. The experimental data in **Paper V** proved that the high molecular weight polymeric mediator penetrates through the thick bacterial cell envelope and can directly enter the respiratory chain to drive the EET reactions. Furthermore, the obtained results disclose the main principles of how to overcome incompatibilities between abiotic electron carriers and microbial metabolism and improve the electronic communications based on MET. This provides essential knowledge for further development and optimization of MESs.

E. faecalis is the very first Gram-positive bacterium with identified EET mechanisms and characterized features of interaction with various mediating compounds. This electrochemical study became possible as a consequence of accumulated knowledge in the physiology of the microbe, availability of a genetic tool for creation of a mutant range with target mutations within the respiratory chain, and material chemistry for synthesis of new mediating compounds of required structures. A mechanistic knowledge of the mediated microbe-electrode exchange is important for improvement and development of MESs, especially within the bioenergy production sector. Furthermore, it essentially adds to understanding the electrical cell-to-cell interconnections, biogeochemical processes in nature and gives a new insight into evolutional aspects of microbial ET.

The high scientific interest in the phenomenon of microbial EET aims to overcome bottlenecks in efficiencies and scaling up of bioelectrochemical systems. Except for a very few model bacterial species, many microbes, though being assigned as electroactive, are not investigated at the level, which would give any significant records on EET processes. The absence of a systematic approach in studying the microorganism-electrode electronic communications causes certain limitations in, e.g., the development of microbial electrochemical techniques, a deeper understanding of global energy and material cycles, intercellular and synergistic interactions between various species. In order to solve such problems one should bring fundamental and applied research groups from different fields of microbiology, electrochemistry, genetic engineering and material science together. The challenging study of the EET mechanisms should become truly interdisciplinary under a common focused interest. Such an approach will allow the creation of a strong background for a further guided optimization and rational use of materials, e.g., electrodes, various surface modifications and mediators. Therefore, in order to advance in applying bioelectrochemical systems, one needs to take a step back and focus on the underlying basis – EET.

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