Engineering microbial electrocatalysis for chemical and fuel production
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In many biotechnological areas, metabolic engineering and synthetic biology have become core technologies for biocatalyst development. Microbial electrocatalysis for biochemical and fuel production is still in its infancy and reactions rates and the product spectrum are currently very low. Therefore, molecular engineering strategies will be crucial for the advancement and realization of many new bioproduction routes using electroactive microorganisms. The complex and unresolved biochemistry and physiology of extracellular electron transfer and the lack of molecular tools for these new non-model hosts for genetic engineering constitute the major challenges for this effort. This review is providing an insight into the current status, challenges and promising approaches of pathway engineering for microbial electrocatalysis.

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Introduction
In microbial bioelectrochemical systems (BES), whole cell microbial biocatalysts act on electrodes to catalyze electrochemical oxidations (at an anode) or reductions (at a cathode). Initially, microorganisms were mainly studied and employed for anodic oxidation of organic substances with the purpose of converting stored chemical energy into electrical energy. These systems are called microbial fuel cells [1–3]. In recent years, the focus has shifted to microbial reductive processes at the cathode. Here, microorganisms can catalyze electrochemical reactions like proton reduction to molecular hydrogen [4,5] or the reduction of carbon dioxide to organics like methane [6,7] or acetate [8,9**]. Thereby, the bioelectrochemical reduction of CO₂ to acetate has been termed microbial electrosynthesis, which holds strong promises for a new concept for biofuel generation [10*,11]. In this process, the central intermediate acetyl-CoA is a versatile building block for a range of chemicals and potential biofuels like ethanol, n-butanol or longer chain fatty acids and alcohols. Metabolic engineering and synthetic biology approaches are expected to open up these new biochemical electrofuel production pathways. However, these new microbial electrocatalytic reactions are not limited to CO₂ as a substrate or to purely reductive reactions. They can also be utilized to perform difficult redox reactions, for example, biochemical or pharmaceutical synthesis that rely on regioselectivity or stereoselectivity of the desired reactions.

There are two major challenges facing this new concept at this point. On the one hand electrocatalytic activity of the studied microorganisms, especially the rate of electron uptake (for reductions), is currently much too low for technical applications. On the other hand, advances in metabolic engineering and synthetic biology for non-model organisms are urgently required to implement the desired biochemical pathways in the respective electroactive microorganisms. Once these molecular challenges will be solved, further engineering hurdles for technical applications will move into the focus. The last section of this article will briefly highlight those future challenges.

Microbial biocatalysts and potential processes
The most well studied electroactive microorganisms to this point are Geobacter sulfurreducens and Shewanella oneidensis, both of which have been mainly studied for bioelectrocitric applications. G. sulfurreducens is a true specialist for extracellular respiration (acetate oxidation with an anode as terminal electron acceptor) and employs specific redox enzymes in its cell envelop (mostly c-type cytochromes) to transfer electrons outside the cell [12–14]. Here, the electrons can directly reduce an extracellular electron acceptor or be further transferred to such over a distance via conductive pili protein nanowires. The mechanism of conductivity of these protein pili is currently under scientific debate [15–18]. S. oneidensis, on the other hand, is more versatile and less specialized in its electroactivity [19]. It is a facultative anaerobic organism, but in the absence of oxygen it can perform a facilitated fermentation of lactate to acetate in presence of an electrode for discharge of surplus electrons. S. oneidensis also employs a series of c-type cytochromes to transfer electrons across the cell envelop [20]. From there, the electrons can be transferred to extracellular electron
acceptors directly or mediated via self-synthesized flavin-type redox shuttles [21,22].

Both organisms also show activity for electron consumption at the cathode [23,24], for example, _G. sulphurreducens_ can reduce fumarate to succinate with a cathode as electron donor. But here the biochemical pathways and the energetics of electron uptake are much less understood. Methanogens and homoacetogens are also highly interesting for reductive processes at the cathode for their ability to reduce carbon dioxide to methane with reducing equivalents from a cathode instead of hydrogen [6,7], and to reduce carbon dioxide to acetate, respectively [8,9].

While in the past decade the main focus in BES microbiology research was on investigating electroactive physiology and screening for new electroactive microorganisms, recent advances are fueling a transition to modifying and optimizing electroactive microbes for target applications. This trend was especially driven by the emergence of the concept of microbial electrobiosynthesis from carbon dioxide and is now expanded to a great variety of potential redox bioconversions both at the cathode (reductions) or at the anode (oxidations) [25,26]. However, current levels of cathodic productivity in microbial electrobiosynthesis are very low. The best of the homoacetogenic organisms known for electrobiosynthesis, _Sporomusa ovata_, was reported to produce about 150 μmoles acetate per day from CO₂ with a cathode at −400 mV versus SHE (standard hydrogen electrode) as sole electron donor [10] at a good coulombic efficiency of 85% (ratio of electrons stored in acetate over electrons consumed from the cathode). This fairly low performance (conversion rate) is still about 10 times higher than the activity of _Clostridium ljungdahlii_ — the most intensively studied microorganism for electrobiosynthesis [8]. This means that for any reasonable application of these organisms as biocatalysts in bioelectrochemical productions, a drastic improvement of the electron transfer rate is prerequisite.

_C. ljungdahlii_ attracts significant research interest for advancing microbial electrobiosynthesis, because its genome is sequenced and genetic engineering efforts for this organism are currently advancing for its applications in autotrophic synthesis gas fermentations of potential fuels and biochemicals [27,28]. The investigation and clarification of the electron uptake physiology, which is currently completely unknown, is one important task of current research. With the low rates of cathodic physiology, it is for example very difficult to determine if cells are growing in this situation. Currently, it is mainly assumed that they are not growing but long-term viable, which would present an important advantage for biocatalytic applications. First engineering tools are currently being developed for _C. ljungdahlii_ (see below) to pave the way for physiological investigations, but progress is fairly slow and existing hypotheses for electron uptake mechanisms could not be resolved over the past years [29]. Another possibility to enhance the rate of cathode to microbe electron transfer, which is currently explored, would be to generate molecular hydrogen at more reduced cathode potentials, which can then mediate electron transfer to the biocatalytic organism. This would also allow utilizing the entire reactor volume for bioproduction, instead of being limited to the two-dimensional electrode. Additionally, the search for naturally more efficient biocatalysts for cathodic bioconversions has just begun and future will show, if we can identify specialists for extracellular electron uptake similar to the high efficiency of _Geobacter_ species for anodic electron discharge.

Thus, it is important for real progress in the field of microbial electrobiosynthesis, that — for both anodic oxidations and cathodic reductions — we always analyze and optimize both ends of the reaction: electron transfer to or from microorganisms and the target bioconversion.

**Challenges to (synthetic) pathway engineering**

The challenge of tailoring an electroactive biocatalyst can be approached by two different strategies (Figure 1). The first strategy includes the transfer of pathways involved in the electroactivity from one organism to an already established biocatalyst. This requires extensive knowledge of the protein functions and the corresponding pathways, which are involved in electron donation to an anode or uptake from a cathode, respectively, including their regulation. These functional proteins have subsequently to be transferred and adapted in order to be functional in a heterologous biocatalytic host. For example, although, there is basic insight in the extracellular transfer of electrons to an anode for _G. sulphurreducens_ and _S. oneidensis_, it is still difficult to define the minimum set of reductases that are required to reproduce extracellular electron transfer. First attempts have been made to tailor _Escherichia coli_ in this direction by installing parts of the extracellular electron transport chain from _S. oneidensis_ (genes mtrCAB) with promising initial results [30]. Although the engineered _E. coli_ showed some activity toward Fe(III) reduction, this hypothetical minimal set of genes for extracellular electron transfer did not enable electroactivity in _E. coli_. Thus, other, so far unknown, components are required to successfully recreate extracellular electron transfer. On the other hand, little to nothing is known about the uptake of electrons from a cathode. Since electroactivity is a complex phenomenon it will prove rather difficult in the short term to engineer a functioning heterologous electroactive biocatalyst.

The second strategy addresses the engineering of an electroactive organism by integrating established or novel biocatalytic pathways into it. This strategy has the advantage that the biocatalytic pathways, with respect to enzyme functions and regulation, are in general much better understood than the electron transfer pathways via
electrodes. Nevertheless, various adaptations have to be considered including promoter activities, the GC content of the expressed genes, enzyme co-factors, energy levels and enzyme functions to successfully implement known pathways in a novel host. While both strategies are worth approaching, engineering of cathodic processes currently solely relies on pathway engineering within the electroactive microbes, due to the limited knowledge regarding electron uptake. But when looking at pathway and strain engineering for cathodic microbial electrocatalysis it should be noted that this field of research is very new and currently involves only a few non-model organisms. This means that the well-established and versatile toolboxes that have been optimized for engineering traditional workhorses like *E. coli* and *Saccharomyces cerevisiae* cannot intuitively be used. Therefore, often basic engineering tools have to be developed and fundamental understanding of the physiology is required, before more traditional strain engineering including the increase of precursor supply and the expression of novel (synthetic) pathways can be successful in these hosts.

**Approaches to (synthetic) pathway engineering**

As mentioned above, two key features for a successful electroactive biocatalyst are an improved utilization of electrons, especially for cathodic processes, and the implementation of robust and selective biochemical pathways. This can only be achieved by expanding the available molecular biological techniques for metabolic engineering and physiological studies. One example of a very promising platform organism for chemical and fuel production that is currently being approached by metabolic engineers is *C. ljungdahlii* [27**]. Although, most tool development and physiological characterization has been done in the context of syngas fermentation or for related organisms like *Clostridium acetobutylicum*, valuable methods have been established, which can also be used

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Two major approaches can be distinguished for engineering microbial electrocatalytic reactions: (a) the capability to exchange electrons with an anode or cathode can be transferred to non-electroactive production hosts. (b) Target biocatalytic pathways can be engineered into native electroactive organisms. The bottom section lists advantages and challenges for each of the two approaches.
to elucidate and improve the electroactivity of \textit{C. ljungdahlii} \cite{31,32,33,34}. A first, very important step, when genetically manipulating organisms, is the transfer of functional DNA elements for creating knockouts or for introducing novel enzyme functions. With the transfer and the expression of a plasmid bearing heterologous genes for n-butanol production via an electrotransformation protocol, first evidence was given that \textit{C. ljungdahlii} might become a valuable, manipulable production host \cite{28}. Subsequently another transformation protocol was developed, which proved to be more efficient and several shuttle plasmids have been tested for functionality \cite{27}. The same transformation protocol has been used for creating the first \textit{C. ljungdahlii} deletion and double deletion mutants via double-crossover homologous recombination with a suicide vector, displaying that the deletion of the ethanol production pathway led to increased production of acetate \cite{27}. Such a gene knockout technique will prove to be very helpful in disrupting competing metabolic pathways that are not desired in an engineered electroactive biocatalyst. The same concept has also been strengthened by the application of a Cre-loxP66/loxp71-based gene removal system to eliminate competing pathways in different \textit{Clostridia} sp. able to utilize syngas \cite{35}. With the simultaneous expression of synthetic pathway genes integrated into the chromosome using a Tn7-based approach, the hosts produced selectively methanol, formate, 2,3-butanediol, n-butanol, ethanol, or acetate \cite{35–39}. These results show that redirecting energy and carbon flow is crucial for the engineering of a superior biocatalyst. Recently, also a first inducible promoter system derived from \textit{Clostridium perfringens} was shown to be useful in \textit{C. ljungdahlii} \cite{34}. This opens the door to controlled gene expression studies in this organism.

Insights into \textit{C. ljungdahlii} energy metabolism show unique autotrophic energy conservation processes via a proton dependent Rnf complex \cite{40}. To get a deeper understanding of the physiology under autotrophic conditions and to see which genes are involved, first gene expression analysis has been performed comparing the transcriptomes of \textit{C. ljungdahlii} grown on CO/CO$_2$ with those grown on fructose \cite{41,42}. A first genome-scale model of the metabolic network of \textit{C. ljungdahlii} has also just become available, which will help to design and connect novel biochemical pathways \cite{43}. Tools such as these can also be useful to elucidate gene functions involved in electroactivity, giving valuable insights as to which genes might be worth manipulating to improve the utilization of electrons.

Besides engineering electroactive organisms on cathodes for reductive production processes, microorganisms might be modified to use anodic reactions to achieve redox balance in fermentation reactions via discharge of excess reducing equivalents. Here, current engineering efforts focus on the well-studied and genetically accessible electroactive host \textit{S. oneidensis}. The concept of balancing fermentations has been confirmed using genetically modified \textit{S. oneidensis} strain heterologously expressing glycerol utilization and ethanol production genes, while linking the fermentation reaction to anodic current discharge to eliminate redox constraints \cite{44}. Besides for bioproduction applications, genetic engineering with \textit{S. oneidensis} also advanced in other areas. Specifically, essential elements of the native electron transport chain of \textit{S. oneidensis} were placed under the control of an inducible promoter, which opens many potential applications in biosensing \cite{45}. Further developments on these engineering successes and development of new molecular toolboxes can then also feed back into the engineering of microbial electrocatalysts for bioproduction.

**Challenges beyond pathway engineering**

We strongly believe that currently pathway engineering, which means in essence lowering the activation overpotential for the target bioelectrochemical reaction, is the primary task for advancing microbial electrocatalysis to usable levels. But once we succeeded with this task, further engineering hurdles will have to be solved to turn this principle into a real technical process. Electrochemical processes are intrinsically two-dimensional, which limits the reaction space in a reactor. Smart electrode design to fill three-dimensional reactors with two-dimensional electrodes will be required. The central electrochemical requirement to keep anode and cathode spacing low and often to separate these electrodes by an ion exchange membrane adds complexity to this task. But these are challenges, which are generally true for BES and internationally much work is ongoing to resolve these technical issues. Other problems are more specific to microbial electroysisynthesis. Thus, when working with obligate anaerobic homoacetogens at a cathode, resulting toxic oxygen formation at the anode might become a problem. Can we substitute for oxygen formation at the anode by utilizing another bioproduction reaction at this electrode? This brings us back to microbial pathway engineering, which might determine if microbial electrocatalysis stays a scientific phenomenon or develops into a technical process.

**Conclusion**

BES have evolved from original environmental target applications like bioelectricity generation or bioremediation to become a promising bioproduction strategy in redox biosynthesis. To achieve this expansion, electroactive microbial biocatalysts need to be tailored for optimum electrocatalysis. However, since the electroactive microbial physiology is still barely resolved and natural electroactive microbes are uncommon hosts for genetic engineering, much fundamental molecular research is required for successful intrinsic and synthetic pathway engineering with these new biocatalysts. First examples
for both oxidative and reductive microbial catalysis indicate that genetic engineering can pave the way for this new concept to become reality.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- - of outstanding interest


This paper shows that microbial electrotransformation is possible with a variety of homoacetogenic bacteria. It is the first mention of C. ljungdahlii as electroactive microorganisms.


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This review provides an early estimate of the potential of microbial electrotransformation for bioproduction.


This editorial text extends the possibilities for microbial electrocatalysis for bioproductions beyond the concept of microbial electrotransformation from carbon dioxide.


First article describing several important fundamental molecular tools for the manipulation of C. ljungdahlii for microbial electrotransformation.


First published work to engineer C. ljungdahlii for biofuel (n-butanol) production from synthesis gas.


Review of cathodic microbial electron transfer physiology with new hypotheses toward electron transfer mechanisms. Still up to date.

This work describes the first efforts to recreate extracellular electron transfer in a non-electroactive host (E. coli).


The modular pMTL plasmid series developed in this work for C. acetobutylicum, presents one of the few available molecular tools also for C. ljungdahlii and has been used successfully by other groups.


Rational synthetic pathway design, might greatly accelerate the efforts of genetic engineering of C. ljungdahlii. A genome-scale metabolic model like this will lay the foundation for this effort.
