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Identification of \textit{Clostridium cochlearium} as an electroactive microorganism from the mouse gut microbiome

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Abstract
Microbial electroactivity, the metabolically relevant transfer of electrons between microorganisms and solid conductors, was first discovered for now well characterized model organisms from hypoxic or anaerobic water or sediment samples. Recent findings indicate that the metabolic trait of electroactivity might as well be important within the microbiome of the mammalian gut. Based on a pre-selection from the mouse intestinal bacterial collection five microorganisms originating from diverse parts of the gut were screened for electroactivity. As there is no marker gene for electroactivity, the ability to synthesize cytochromes and metabolize redox mediators was studied \textit{in silico}. \textit{Clostridium cochlearium} showed highest electroactivity and \textit{Lactobacillus reuteri} as well as \textit{Staphylococcus xylosus} show putative electroactivity, as well. The maximum current density of \textit{C. cochlearium} of 0.53 ±0.02 mA cm\textsuperscript{-2} after only 5.2 hours of incubation was clearly linked to growth and glucose consumption. Cyclic voltammetric analysis on \textit{C. cochlearium} revealed a formal potential of the extracellular electron transfer (EET) of +0.22 ±0.05 V versus Ag/AgCl sat. KCl (and +0.419 V versus SHE) and indicates that EET is not based on biofilm formation, but the involvement of either redox-active molecules or planktonic cells. The potential of the gut as habitat for electroactives and their physiological role are discussed.

Keywords
gut microbiome, electroactive bacteria, extracellular electron transfer, electrochemical screening, microbial electrochemistry
Microbial electrochemistry is a subfield of bioelectrochemistry devoted to the study and engineering of electrochemical interactions of microorganisms and electrodes [1]. In the last decades the increasing research in microbial electrochemistry was mainly driven by the interest in future applications at the nexus of electric power generation and storage as well as (bio)chemical conversions for production of value added chemicals [2,3]. These applications range from biosensors, wastewater treatment to synthesis of chemicals that are summarized under the umbrella of microbial electrochemical technologies [1]. The foundation of all primary microbial electrochemical technologies are electroactive microorganisms [4–7]. The interaction of electroactive microorganisms with the electrode is of Faradic nature and termed extracellular electron transfer (EET) [8]. The detailed mechanisms of EET are increasingly deciphered and lively debated, but in essence EET allows coupling the microbial metabolism with the external current flow. This is studied in devices termed bioelectrochemical systems (BES). Evolutionary it can be assumed that EET served as metabolic trait enabling the exploitation of insoluble terminal electron acceptors (TEA) like minerals as well as the trophic interactions based on transfer of electrons between microorganisms. The latter being now termed inter- or intraspecies electron transfer (IET). Using EET the TEA can be reached via two possible means: direct electron transfer (DET) and mediated electron transfer (MET). For DET the electroactive microorganisms rely on the immediate physical contact with the TEA, i.e. a mineral or electrode. For MET, a mediator, i.e. a redox active molecule that can be reversibly oxidized or reduced, is secreted by the microorganism (endogenous mediators) or added to solution (exogenous mediators). MET has been already described for several compounds, including flavins and its derivatives, phenanzines and viologens. These modes of EET can be found in the archetypes for electroactive bacteria (EAB) that are Geobacteraceae and Shewanellaceae [4,9–14]. However, there is also increasing awareness that a great diversity of microorganisms can perform EET [5]. Thus, not only the question arises "How to precisely define an electroactive microorganism?" [6], but especially it poses the intriguing question "What is the evolutionary trigger for electroactive microorganisms?".

In the last years it was shed light on the fact that, for instance, electroactive microorganisms may have played an important role in the early phase of the formation of the Earth and contribute significantly to geochemical nitrogen and carbon cycles [15–19]. Very recently, it was also speculated that the gut microbiome harbors electroactive microorganisms and that they play an important role therein [20]. The mammalian gut system is a complex, diverse and yet unique microbial habitat. Microbial substrates (i.e. electron donors and reduced carbon sources) are available in high concentrations, being in contrast to other habitats, like soil [21,22]. Further, water supply, ambient temperature and a mainly reduced environment create favorable growth conditions for microorganisms using other TEA than oxygen [23]. The functionality of the gut microbiome, i.e. the microbial community in the gut, is of highest importance for its host. Nutrients and cofactors, such as simple monomers of carbohydrates, vitamins, short chain fatty acids and hormones are products of the microbiome [24–26]. Essentially, microbial metabolism supplies its host with fuel for catabolism and anabolism, but only recently it has been shown that these compounds, their concentration and the diversity of the microbiome have severe impacts on human health, as well [27,28]. The gut microbiome was shown to be highly diverse and stratified. Stratification means that the microbial communities in the different gut sections vary significantly. This is
due to physical-chemical and anatomical conditions therein and mainly due to chyme (i.e. semi-fluid mass of partly digested food) entering the duodenum from the stomach, creating a pH and O2 gradient throughout the intestine [29]. Furthermore, the intestinal wall has a high degree of folding and thus creates further micro-environments [23]. In summary this creates several ecological niches, characterized by the availability of substrate and TEA. Further community shaping factors are proximity to the epithelium, concentration of antimicrobials and antibodies as well as localization alongside the length of the gut.

This diversity of microenvironments is resembled in the diversity and abundance of microorganisms inhabiting the niches mentioned above. Fast-growing and metabolically diverse microorganisms, such as members of Firmicutes, outcompete others (Bacteroidetes) in the proximal part, as they can more efficiently take up nutrients and cope with rather unfavorable conditions, for instance high concentrations of antimicrobials and bile acid [23]. In turn the distal part of the gut is much more densely colonized [30]. The overall microbial gut community consists mainly of Bacteroidetes and Firmicutes. The latter being highly abundant and diverse, but their physiology is not yet completely understood, as most of them are still uncultivable [31]. This complex interplay of the mammalian host, chemistry and microbiology and especially the creation of microbial food-webs based on carbon compounds, hydrogen or maybe even IET is only begun to be understood [32–34]. The speculation that IET and hence EET may play a role in gut microbiomes is further fueled by the finding that some electroactive microorganisms are also present in the gut microbiome. One such microorganism is *Faecalibacterium prausnitzii* [35]. Under anaerobic conditions *F. prausnitzii* produces butyrate from complex carbon sources that serves as energy and carbon source for the epithelial cell [36,37]. When no aerobic respiration is possible *F. prausnitzii* can switch to riboflavin as TEA [38]. This is well in-line with experiments showing electroactivity of *F. prausnitzii* using phenazine as mediator for EET [39]. Thus *F. prausnitzii* may use EET to overcome oxygen limitation. But the ability to make use of phenazines can as well result in increased biofilm formation and thus in virulence [40]. This was shown for *P. aeruginosa*, a gut- and lung-associated human pathogen, that uses the phenazine pyocyanin [41]. These phenazines as well have shown to be excellent mediators for enabling EET of *P. aeruginosa* [42,43]. Improved biofilm formation has as well been shown for another pathogenic gut bacterium, *E. faecalis*, which exploits EET to shuttle electrons to insoluble TEA being harvested through fermentation [44,45].

In order to investigate the gut microbiome as potential habitat of electroactive microorganisms selected gut associated microorganisms resembled in the mouse intestinal bacterial collection (miBC) [46] were characterized. This characterization was performed using bioelectrochemical cultivation by chronoamperometry, cyclic voltammetry and in-silico screening for potential marker genes on electroactivity. The selection of the miBC was derived by Lagkouvardos et al. (2016) [46], who made a significant effort to isolate and cultivate microorganisms from diverse parts of the murine intestine. The miBC selection is representative of the mouse gut microbiome, it is characterized by a distribution of phyla, and hence similar to the ones found in humans [47]. Hence this selection does serve as an excellent model base for further exploring the gut microbiome as habitat of electroactive microorganisms.

2. Material & Methods

All potentials provided in this study refer to the Ag/AgCl sat. KCl reference electrode (+197 mV vs SHE), if not indicated otherwise.
2.1. Chemicals, strain selection, and culture maintenance

All chemicals were of at least analytical grade and were supplied from Carl Roth GmbH (Karlsruhe, Germany) and Merck KGaA (Darmstadt, Germany). De-ionized water (Millipore, Darmstadt, Germany) was used to prepare the microbial growth media, substrate and buffer solutions.

Five out of 100 microorganisms were chosen from the mouse intestinal bacterial collection (miBC) [46] for electrochemical screening (Table 1).

The selection was based on two parameters: i) microorganisms only belonging to biosafety level (BSL-1) were selected; thereof ii) the microorganisms having a fully annotated genome available in the National Center for Biotechnology Information database (NCBI, Rockville, USA) were chosen. The selected microbial strains (see Table 1) were purchased from German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany) and cultured for maintenance in batch at 37 °C in the recommended media.

Table 1. Microbial strains selected from the miBC for electrochemical screening. Maintenance cultivation was carried in out in DSMZ media and electrochemical cultivation in minimal media as listed.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DSMZ strain number</th>
<th>DSMZ Medium for maintenance</th>
<th>DSMZ Medium for maintenance</th>
<th>Medium for electrochemical screening</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium cochlearium</td>
<td>DSM 29358</td>
<td>DSM 104</td>
<td></td>
<td>Firmicutes Minimal Medium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[48,49]</td>
</tr>
<tr>
<td>Lactobacillus reuteri</td>
<td>DSM 28673</td>
<td>DSM 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus xylosus</td>
<td>DSM 28566</td>
<td>DSM 92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>DSM 26127</td>
<td>DSM 1669</td>
<td></td>
<td>Verrucomicrobia Minimal Medium&lt;sup&gt;b&lt;/sup&gt;</td>
<td>[49,50]</td>
</tr>
<tr>
<td>Bacteroides vulgatus</td>
<td>DSM 28735</td>
<td>DSM 339</td>
<td></td>
<td>Bacteroides Minimal Medium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[49,51]</td>
</tr>
</tbody>
</table>

<sup>a</sup>with 0.5 % glucose (w/v); <sup>b</sup>with 0.5 % N-acetylglucosamine.

For maintenance of anaerobic cultures, weekly subcultivation was performed in 200 mL serum bottles containing 100 mL medium in N<sub>2</sub> atmosphere. The serum bottles were incubated at 37 °C.

2.2. Microbial electrochemical screening

The electrochemical screening of microorganisms was conducted in BES being two-chamber electrochemical cells made of 100 mL Duran glass bottles with 95 mL working volume. The working electrode (WE) and counter electrode were graphite rods (CP Handels GmbH, Wachtberg, Germany) with a geometric surface area of 4.3 cm<sup>2</sup> linked to the potentiostat via titanium wire (Ø 0.5 mm, Goodfellow, Cambridge, England). The WE and reference electrode (RE, Ag/AgCl sat. KCl reference electrode (+197 mV vs. SHE, Xylem Analytics Germany Sales GmbH & Co/ Meinsberg Sensortechnik GmbH, Germany)) were assembled in a butyl rubber stopper and chemically sterilized (70 % Ethanol, 100 mM H<sub>2</sub>SO<sub>4</sub>). The autoclaved counter electrode chamber of 15 mL maximum volume was aseptically mounted to the butyl rubber stopper. Thus, the counter electrode was physically separated but ionically connected to the WE chamber via a membrane (fumasep® FKE, Fumatech, Bietigheim-Bissingen, Germany). This was done in order to prevent H<sub>2</sub> intrusion to the anaerobic WE chamber from H<sub>2</sub> evolution on the counter electrode.
Before and after each experiment the graphite electrode surfaces were cleaned with sandpaper (WetorDry P1200, 3M, Minnesota, USA). The WE chamber was closed gas tight and flushed with sterile N₂ (2 bar, 30 min) to create an anoxic environment. The counter electrode chamber was filled with 10 mL of minimal medium without carbon source.

All experiments were carried out under strictly anaerobic conditions and potentiostatic control using a multi-channel potentiostat/galvanostat (MPG-2/VSP, BioLogic Science Instruments, Claix, France) at 37 °C (Unihood Unequip, Planegg, Germany) and stirred at 120 rpm (2mag, München, Germany).

For inoculation of the BES, a pre-culture was prepared. Therefore, 5 mL of anaerobic maintenance culture or one colony forming unit (CFU) from the agar plate of an aerobic strain were inoculated to 100 mL of the respective medium for electrochemical screening (Table 2, medium composition listed in Table S1, experimental setup Fig. S1). After 24h the BES were inoculated with 5 ml of the pre-culture.

Control measurements were either performed using the identical setup without potential applied (open circuit voltage, OCV control) or without inoculation (negative control). Electrochemical cultivation was performed using chronoamperometry (CA) at +0.5 V in order to provide maximum thermodynamic driving force for anodic activity.

Using cyclic voltammetry (CV) three cycles were recorded at the beginning (t₀) and end (tₚₐₐₜ) of the experiment with a scan rate of 1 mV s⁻¹ and only the 3rd cycle (being steady-state) used for further analysis. The CVs of *C. cochlearium* and *L. reuteri* were performed with a scan range from -0.5 to +0.65 V. The CVs of the other microorganisms ranged from -0.65 to +0.65 V to cover a broader spectrum for potential electrochemical interaction with the electrode.

2.3. Electrochemical in-depth study of *C. cochlearium*

In order to gain deeper insight into the electrochemical behavior of *C. cochlearium* another set of experiments was designed, applying the electrochemical cultivation techniques as mentioned above. After the initial CVs, chronoamperometry at +0.5 V was performed for 3.7 hours, thereafter turnover CVs were recorded. Subsequently, one set of BES was further cultivated using chronoamperometry, while the other set was split for analysis by CV as follows (and also depicted in Fig. S2): I) the working electrode was moved to a new 100 mL bottle containing 95 mL of fresh minimal medium; II) the microorganisms, i.e. the cell pellet obtained by centrifuging the suspension (3000 x g, 20 minutes, 4°C), was anaerobically re-suspended in 95 mL fresh medium and transferred to a new sterile BES; III) the supernatant derived from centrifugation was transferred to another sterile BES.
2.4. Chemical and microbial analysis

Optical density ($OD_{600}$) was measured with a spectral photometer (UViLine 9400, SI Analytics, Mainz, Germany) at a wavelength of 600 nm. For pH measurements at $t_0$ and $t_{end}$ a pH meter was used (LaquaTwin B-712, Horiba Scientific, Bensheim, Germany). High-performance-liquid-chromatography (HPLC) was performed. After the centrifugation (13000 x g, 10 minutes) supernatant was diluted (1:5) and filtered (0.2 µm pore size, nylon, Sartorius, Göttingen, Germany) before HPLC analyses (Shimadzu Scientific Instruments, Kyoto, Japan) using a HiPlex H column (300 x 7.7 mm, 8 µm pore size, Agilent Technologies, Santa Clara, USA) with 5 mM H$_2$SO$_4$ as mobile phase (0.5 mL min$^{-1}$ and 50 °C) and a refractive index detector (RID-10A). The signal was calibrated for glucose, in the range of 0.02 g L$^{-1}$ to 1 g L$^{-1}$, and for lactate, formate, butyrate and acetate in the range of 0.02 g L$^{-1}$ to 0.5 g L$^{-1}$.

2.5. Bioelectrochemical calculations

In order to determine the efficiency of microbial current production, the Coulombic efficiency (CE) was calculated (Eq. 1).

$$CE = \frac{n_{e-}(\text{real})}{n_{e-}(\text{theoret})} \times 100 \quad (1)$$

The molar amount of electrons ($n_{e-}(\text{real})$) is calculated from the total charge ($q_{tot}$ C) harvested during chronoamperometry divided by the Faraday constant ($F = 96485.33 / \text{C mol}^{-1}$) (Eq. 2).

$$n_{e-}(\text{real}) = \frac{q_{tot}}{F} \quad (2)$$

The theoretical amount of electrons ($n_{e-}(\text{theoret})$) is calculated from one of the possible redox reactions based on the amount of degraded glucose converted to butyrate (Eq. 3) or being completely oxidized to CO$_2$ (Eq. 4).

$$\text{C}_6\text{H}_12\text{O}_6 \rightarrow \text{C}_4\text{H}_7\text{O}_2^- + 2\text{CO}_2 + 5\text{H}^+ + 4e^- \quad (3)$$
$$\text{C}_6\text{H}_12\text{O}_6 + 6\text{H}_2\text{O} \rightarrow 6\text{CO}_2 + 24 \text{H}^+ + 24e^- \quad (4)$$

Please note that that due to the complex media components (that may serve as electron donors as well as acceptors) an exact CE can only be provided on a carbon balance.

2.6. Pathosystems Resource Integration Center (PATRIC)

The data for in-silico analysis were retrieved from The Pathosystems Resource Integration Center (PATRIC, version 3.5.21, latest used 31.08.18) It is an all-bacterial bioinformatics resource center [52] that is cross-linked to KEGG (Kyoto Encyclopedia of Genes and Genomes).

For this work, the genome sequences of the microorganisms investigated, together with those of model-organisms for electroactivity (Geobacter sulfurreducens and Shewanella oneidensis) were grouped and saved in the cloud-space of PATRIC database. Based on this set of genetic information, different analyses were conducted. To date, several microbial features, such as cytochromes and pili, are described to be involved in DET and with that can be considered to be putative marker genes for electroactivity. The presence of these putative marker genes was investigated in the microorganisms under study. MET was mainly described for flavins [9], thus their
derivatives and other vitamins could be assumed to be involved as mediators for electron transfer. Hence, their presence in the microbial genome was as well analyzed. Putative marker genes (Table 2) for DET were defined and evaluated regarding presence and abundance (#/-). Furthermore, pathways for mediator synthesis and transformation were evaluated, to elucidate if the microorganisms are able to self-synthesize (1/0) and metabolize (*) them.

Table 2. Selected putative marker genes and electrochemical mediators related to direct and mediated microbial extracellular electron transfer (DET and MET), according to literature.

<table>
<thead>
<tr>
<th>Putative genes and metabolites related to microbial electroactivity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putative marker genes for electroactivity (DET)</td>
<td></td>
</tr>
<tr>
<td>Bacterial pili (pilin)</td>
<td>[53]</td>
</tr>
<tr>
<td>Conductive pili (PilA)</td>
<td>[53]</td>
</tr>
<tr>
<td>Nanotubes (YmdB)</td>
<td>[53]</td>
</tr>
<tr>
<td>Cytochrome-containing enzymes</td>
<td>[4]</td>
</tr>
<tr>
<td>Ferredoxin-containing enzymes</td>
<td>[4]</td>
</tr>
<tr>
<td>Vitamins as possible electrochemical mediators (MET)</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>[54]</td>
</tr>
<tr>
<td>Thiamine</td>
<td>[54]</td>
</tr>
<tr>
<td>Riboflavine</td>
<td>[11,38]</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>[54]</td>
</tr>
<tr>
<td>Folate</td>
<td>[54]</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>[54]</td>
</tr>
<tr>
<td>Retinol</td>
<td>[54]</td>
</tr>
</tbody>
</table>

2.7. Data analysis and statistics

All BES were built in three fully independent biological replicates being used for calculations of standard deviation. For HPLC analysis three technical replicates were prepared thereof. Calculations of maximum current intensity ($i_{max}$), maximum current density ($j_{max}$), total charge ($q_{tot}$) and glucose concentration ($c_{glucose}$) were then depicted as the arithmetic mean with $n \geq 3$ and are provided as mean $\pm$ standard deviation. $OD_{600}$ was measured in one representative biological replicate, with three technical replicates.

Potentiostat data analysis – including CA and CV - was done using OriginPro9 (OriginLab Corporation, Northampton, MA, United States). Calculation of formal potentials ($E_f$) was based on the arithmetic mean of peaks in the first-derivative of the turnover CV.

3. Results

3.1. Microbial electrochemical screening

For electrochemical screening chronoamperometry under anaerobic conditions at +0.5 V was performed for each strain (Fig. 1 and Table 3). *C. cochlearium* showed a current peak of 0.53 ±0.02 mA cm$^{-2}$ already after only 5.2 h. This is about tenfold higher than the $i_{max}$ recorded for *L. reuteri* and *S. xylosus* after 20 - 25 h of bioelectrochemical cultivation. After the $i_{max}$ (Table 3) was reached, the
signal lowered to a value close to zero and the bioelectrochemical cultivation was stopped. Neither *A. muciniphila* nor *B. vulgatus* produced a significant current. All negative controls showed no microbial growth and all OCV controls no current production, but microbial growth. Further, please note that chronoamperometric cultivation at -0.5 V was also tested but no current was detected for all selected strains (see also Figure SI 1).

![Graph](image_url)

**Fig. 1.** Current production during chronoamperometric cultivation at +0.5 V of the selected microorganisms at 37 °C and 120 rpm. One representative dataset per strain is shown until 40 h of incubation and no significant current was measured thereafter. *C. cochlearium* (black, solid line), *S. xylosus* (grey, solid line), *L. reuteri* (black, dotted line), *A. muciniphila* (black, broken line), *B. vulgatus* (grey, broken line). Further, negative (i.e. abiotic) controls also did not show current production.

<table>
<thead>
<tr>
<th>Strain</th>
<th>j&lt;sub&gt;max&lt;/sub&gt; / mA cm&lt;sup&gt;-2&lt;/sup&gt;</th>
<th>q&lt;sub&gt;tot&lt;/sub&gt; / C</th>
<th>t / h</th>
<th>pH&lt;sub&gt;end&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium cochlearium</em></td>
<td>0.53 ±0.02</td>
<td>44.28 ±0.93</td>
<td>40.0</td>
<td>7.03</td>
</tr>
<tr>
<td><em>Lactobacillus reuteri</em></td>
<td>0.05 ±0.02</td>
<td>16.14 ±3.06</td>
<td>40.0</td>
<td>5.97</td>
</tr>
<tr>
<td><em>Staphylococcus xylosus</em></td>
<td>0.04 ±0.01</td>
<td>20.70 ±2.57</td>
<td>40.0</td>
<td>5.87</td>
</tr>
<tr>
<td><em>Akkermansia muciniphila</em></td>
<td>0.01 ±0.00</td>
<td>10.05 ±0.40</td>
<td>40.0</td>
<td>5.53</td>
</tr>
<tr>
<td><em>Bacteroides vulgatus</em></td>
<td>0.00 ±0.00</td>
<td>5.21 ±3.12</td>
<td>40.0</td>
<td>5.73</td>
</tr>
</tbody>
</table>

Table 3. Average (n=3, mean ±standard deviation) of maximum current production (j<sub>max</sub>) and total charge (q<sub>tot</sub>) of chronoamperometric cultivation (duration: t/h) of the five selected microorganisms. pH was measured at the end of each experiment.

To gain mechanistic information CVs were recorded at t₀ and t<sub>end</sub> (Fig. 2, showing the third cycle of each CV). The CVs showed only for *C. cochlearium* and *L. reuteri* a difference between t₀ and t<sub>end</sub>. This might point towards the capability to be electroactive under the conditions...
tested, and hence would be in accordance with CA (Table 3). The CVs recorded at $t_{\text{end}}$
show signals for *C. cochlearium* (Fig. 2A); starting at a potential of around -0.18
±0.06 V and for *L. reuteri* (Fig. 2B) starting at a potential of -0.04 ±0.01 V. For more
positive potentials the current slightly increases further for both microorganisms, which
should not be overinterpreted and needs a more thorough analysis. On the contrary,
the CVs recorded for *S. xylosus*, producing the same total charge as *L. reuteri*, did not
show any remarkable signal. The CVs on BES with *A. muciniphila* and *B. vulgatus* (Fig.
2C, 2D and 2E) confirmed the absence of redoxactive centers, which were also not
present in the pure media (see Figure S3). *Firmicutes* minimal medium showed a
vague signal of a redoxactive center with a formal potential of -0.01 at $t_0$, but it was
gone by the time the experiment ended ($t_{\text{end}}$). It can be excluded that signals obtained
from *C. cochlearium* or *L. reuteri* are due to this.
Fig. 2. Cyclic voltammetry (CV) recorded for reactors with selected microbial strains at the beginning ($t_0$, broken line) and at the end ($t_{end}$, solid line) of the electrochemical cultivation (see Fig. S1), scan rate: 1 mV s$^{-1}$; 3rd cycle of one representative CV per strain.
3.2. Electrochemical in-depth study of *C. cochlearium*

During the electrochemical screening, *C. cochlearium* was found to be the most electroactive microorganism (see Table 3) and was thus subjected to in-depth studies. Therefore, during electrochemical cultivation the current production was gradually assessed with microbial growth (in terms of $OD_{600}$) and glucose consumption (Table 4). The current production was directly linked with planktonic growth ($OD_{600}$ measurement, see also Fig. S4) and current production was only occurring while microorganisms were in their exponential phase.

After initial cultivation for 5.5 h current production reached 1.87 ±0.29 mA ($j_{\text{max}} = 0.44 ±0.07$ mA cm$^{-2}$) and the turnover CV was recorded, showing an $E_f$ of +0.22 ±0.05 V. Growth reached stationary phase and current production did not increase again during further 13 h of incubation (Table 4). This is also in line with the glucose consumption, as 6.01 ± 0.96 % of glucose was degraded after 7.5 h of incubation. In the BES incubated for 20.5 hours the degradation was only slightly increased to 6.59 ± 1.91 % (Table 4), meaning that only 0.58 % of glucose were degraded between 7.5 and 20.5 hours of incubation. It can be concluded that glucose was mainly degraded while the bacteria were producing current in the exponential growth phase. This further clearly evidences the electroactivity of *C. cochlearium*.

Table 4. Chronoamperometric cultivation of *C. cochlearium*: average of $OD_{600}$, total charge ($q_{\text{tot}} / C$) and glucose concentration ($c_{\text{glucose}} / \text{mM}$) during 0, 7.5 and 20.5 h of incubation. Thereof, the consumption of mole of carbon ($\Delta[C \text{-mol}] / \text{mM}$) derived from glucose into fermentation products was calculated. No correlation between glucose consumption and formation of fermentation products could be observed.

<table>
<thead>
<tr>
<th>t / h</th>
<th>$OD_{600}$</th>
<th>$q_{\text{tot}} / C$</th>
<th>$C_{\text{glucose}} / \text{mM}$</th>
<th>$\Delta[C \text{-mol}] / \text{mM}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>0</td>
<td>0.07 (±0.0)</td>
<td>0 (±0.0)</td>
<td>26.84 (±0.36)</td>
<td>0.0 (±0.0)</td>
</tr>
<tr>
<td>7.5</td>
<td>0.41 (±0.0)</td>
<td>34.05 (±4.66)</td>
<td>25.10 (±0.11)</td>
<td>-10.12 (±2.74)</td>
</tr>
<tr>
<td>20.5</td>
<td>0.55 (±0.0)</td>
<td>48.77 (±9.15)</td>
<td>25.34 (±0.29)</td>
<td>-9.26 (±1.33)</td>
</tr>
</tbody>
</table>

The CE was calculated based on assuming either the fermentation of glucose to butyrate (Eq. 3) or the full oxidation to CO$_2$ (Eq. 4) as 5.77 ±0.84 % and 0.96 ±0.14%, respectively. However, the possible consumption of yeast extract and peptone that could not be quantified further impairs the determination of CE.

To shed further light on the mode of electron transfer, CV analysis as follows was performed. The BES was disassembled after recording the turnover CV and separate CVs of I) the (potentially) biofilm covered WE, II) microorganisms in the obtained cell pellet from the planktonic phase and III) the supernatant thereof were performed (see also 2.3. and Figure S2). Fig. 3A shows their obtained CVs in comparison with the CV recorded in the original BES and their first derivatives.
No electrochemical signal was obtained for I) the WE. Thus, we conclude that the current production was therefore not linked to electrode attached molecules or bacteria (Fig. 3C). Consequently, one can deduce that planktonic bacteria and metabolites secreted during growth are very likely responsible for electroactivity. This was confirmed by the CVs recorded from II) the microorganisms and III) the supernatant. Both of them showed a voltammetric signal with similar inflection points and hence formal potentials, $E_f$: II) $+0.17 \pm 0.00$ V (Fig. 3D) and $+0.16 \pm 0.04$ V (Fig. 3E). Obviously, the $E_f$ of this turnover CVs are far more positive than the signals obtained for the non-turnover CV of *C. cochlearium* (Fig. 2A), but noteworthy, the onset potentials of the CVs shown in Fig. 3A of about $-0.2$ V is very similar. The $E_f$ in Fig. 3D and Fig. 3E are also similar to that derived from CVs of the original BES ($+0.22 \pm 0.05$ V; Fig. 3B, Table S2). Thereby current production was twice as high at a potential of $+0.5$ V when comparing III and II, which might be due to a higher concentration of mediators secreted by further actively growing microorganisms. The deviations between $E_f$ of the original BES and the cell pellet/supernatant of approx. 0.05 to 0.07 V can thereby be assigned to differences in the physical-chemical environment of the fresh and used solution, e.g. minor pH-changes.
It can be clearly stated that with *C. cochlearium* a so far unknown electroactive bacterium was found. It did not form an electroactive biofilm on the electrode, but either performed DET without permanent attachment on the electrode or MET. Furthermore, it can be concluded that current production is linked to an actively, planktonically growing culture of *C. cochlearium* as can be deduced from results of *OD*$_{600}$.
measurement and glucose consumption. Further experiments should point at deciphering the mode of EET, e.g. identifying possible mediators, or analyze the relation of growth and current production as a function of growth medium composition.

3.3. In-silico analysis

In parallel to the above described experimental study a bioinformatics assessment was performed. Therefore, marker genes previously described to be involved in the EET mechanisms and vitamins that could serve as mediators for MET were selected (Table 2) and their presence in the genome of the selected microorganisms (Table 1) and in the model organisms G. sulfurreducens and S. oneidensis was evaluated through PATRIC database.

Table 5 Abundance of putative marker genes and possible electrochemical mediators in the microorganisms studied and in the model EAB G. sulfurreducens and S. oneidensis. The data were taken from PATRIC and the amount of encoded marker genes was counted if present (#/-). Microbial ability to self-synthesize (1/0) and metabolize (*) potential electrochemical mediators was investigated.

<table>
<thead>
<tr>
<th>Putative marker genes for electroactivity</th>
<th>G. sulfurreducens</th>
<th>S. oneidensis</th>
<th>C. cochlearium</th>
<th>L. reuteri</th>
<th>S. xylosus</th>
<th>A. muciniphila</th>
<th>B. vulgatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochromes [4]</td>
<td>122</td>
<td>90</td>
<td>2</td>
<td>6</td>
<td>14</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Pilin [53]</td>
<td>1</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PilA [53]</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ferredoxin [4]</td>
<td>33</td>
<td>11</td>
<td>21</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>YmdB [53]</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Phosphodiesterase [53]</td>
<td>5</td>
<td>17</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Metabolism of selected electrochemical mediators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin [54]</td>
<td>1*</td>
<td>1*</td>
<td>1</td>
<td>1</td>
<td>1*</td>
<td>1</td>
<td>1*</td>
</tr>
<tr>
<td>Riboflavin [11,38]</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Folate [54]</td>
<td>1</td>
<td>1*</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Pantothenate [54]</td>
<td>1*</td>
<td>1*</td>
<td>1</td>
<td>1</td>
<td>1*</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>Retinol [54]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Thiamine [54]</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1*</td>
<td>0*</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine [54]</td>
<td>0</td>
<td>1*</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

It was found that C. cochlearium had a high number of genes encoding ferredoxin containing enzymes (21) compared to the model-organisms G. sulfurreducens (33) and S. oneidensis (11) (Table 5).

On the contrary, only two genes encoding for cytochromes were found in the genome of C. cochlearium, compared to 122 genes in the G. sulfurreducens genome. That result may justify the absence of an electrochemical signal in Fig. 3C. L. reuteri and S. xylosus did not show elevated amounts of putative marker genes for electroactivity. C. cochlearium, L. reuteri and S. xylosus, like all other microorganisms studied and the model-organisms, are able to synthesize riboflavin (Table 5). However, the $E_i$ of the putative mediator or electroactive center in C. cochlearium (+0.22 V vs. Ag/AgCl (sat.
KCl) being $+0.42$ V vs. SHE) is different to that of riboflavin (-$0.22$ V vs. SHE [54]) and all other vitamins tested. Although *A. muciniphila* and *B. vulgatus* did not produce current under the conditions tested, they harbor certain putative marker genes. One may now speculate that these microorganisms are not electroactive *per se*, or we did not find them to be electroactive which can be due to the fact that the genes were less expressed in the conditions applied. Further, the exploitation of genetically engineering microorganisms, e.g. [55], and engineering the interface of microorganisms and electrodes might be options to be considered, e.g. [56].

4. Discussion

It was demonstrated that three out of five microorganisms (*C. cochlearium, L. reuteri* and *S. xylosus*) from the mouse intestinal bacterial collection showed a current production when being cultivated at $+0.5$ V. The in-depth characterization of *C. cochlearium* shows that the current production is linked to planktonic growth and indicates that *C. cochlearium* does not attach to the electrode, but that current is mainly due to the presence of actively growing microorganisms and/or a secreted mediator.

The average maximum anodic current density was $0.53 \pm 0.02$ mA cm$^{-2}$ using a graphite rod electrode at $+0.5$ V. In relation to other electroactive microorganisms and when not considering the exceptional *Geobacteraceae*, this is a remarkable value. For instance, Grobbler *et al.* [57] reported maximum current densities of $0.025 \pm 0.002$ mA cm$^{-2}$ after 18.5 hours of incubation of *Shewanella* cultures grown on a carbon cloth. Furthermore, *C. cochlearium* showed current production already from the beginning of cultivation using CA, i.e. after inoculation of an active pre-culture and performing three initial CV scans. The current further increased when the microorganisms entered exponential phase after 3 hours and reached maximum current and cell density after 5.5 hours of bioelectrochemical cultivation.

The mode of interaction with electrochemical mediators in *S. oneidensis* is based on a cytochrome-based enzyme complex, mtrABC, which is responsible for reduction of flavins that are present in high concentrations [11,58]. This complex is neither present in *C. cochlearium* nor any of the other bacteria tested. Sequence similarity to the newly described EET locus in gram-positive bacteria [20], which is also based on flavins, could not be found in any of the microorganisms investigated. The detected $E_f$ of $+0.22 \pm 0.05$ V of the redoxactive center or secreted mediator in *C. cochlearium* cultures did not match any of the $E_f$ described for selected electrochemical mediators, such as flavines and certain vitamins (Table 5).

For other *Clostridiaceae* than *C. cochlearium* cathodic electroactivity, i.e. current consumption, is reported [59]. *Clostridia* reported to take up electrons from cathodes are *C. pasteurianum, C. ljungdahlii* and *C. aceticum* according to Choi *et al.* [59]. In their study, they found that *C. pasteurianum* is able to directly consume electrons from the electrode without any mediator involved [59]. Here it is of note that the class *Clostridia* experienced several reclassifications, and microorganisms were re-assigned and out-grouped of this class. Still, inconsistencies in terms of classification within this class can be found [60] and it might be possible that these are also accounting for some of the *Clostridia* investigated in microbial electrochemistry. It is therefore not surprising that *C. cochlearium* behaves differently than the *Clostridiaceae* listed above.

Especially, considering the fact that up to the general knowledge the metabolic trait of electroactivity is also not directly related to phylogenetic similarity [5]. *In-silico* analysis revealed that *C. cochlearium* harbors genes for ferredoxins and as well encodes YmdB. Both of them are considered essential for nano-tube formation
between bacteria. YmdB is proposed to be the genetic feature for nano-tube formation itself, while ferredoxin serves as mediator for electron transfer between the species connected via the nano-tube [53]. This mechanism has been previously demonstrated for Clostridium acetobutylicum and Desulfovibrio vulgaris, and it would be of great interest, if it is related to a gut microbiome microorganism such as C. cochlearium. However, the found $E_f$ of the secreted mediator or redoxactive center in C. cochlearium has a formal potential of $+0.22 \pm 0.05$ V (being $+0.42$ V vs. SHE), which lies not in the range of $E_f$ described for ferredoxin (-0.42 V) [61].

Overall, various members of the Clostridia clusters XIVa and IV are described to be highly important for the functionality of the distal gut part [62]. Closely associated to the mucosa, their presence is in different ways linked to host health. As commensals they defend the gut from colonization through pathogens, known as colonization resistance [63]. As strict anaerobes their presence in the distal parts leads to the production of important fermentation metabolites that serve as energy source for the host (short chain fatty acids) [37] and its epithelial cells (butyrate) [64]. A. muciniphila and B. vulgatus did not produce current in the conditions tested in this study. Therefore, the answer to the question why some microorganisms are electroactive while others are not, needs further and broad in-silico analysis. Experiments including the design of new pipelines should aim at finding out what kinds of combinations of the presence of the marker genes and metabolic pathways related to electroactive capability are present.

But even little amounts of current measured can be of significant importance in the gut. For instance, understanding the mode and impact of EET by C. cochlearium and other gut commensals could support the development of prebiotics. Here approaches based on the electrochemical screening of gut samples, as e.g. recently shown by Naradasu et al. [65] on one fecal sample, are also highly promising.

5. Conclusions

Based on a selection of five microorganisms from the mouse intestinal bacterial collection and their experimental evaluation in standardized electrochemical cultivation as well as bioinformatic assessment we have shown that one out of five strains, C. cochlearium, showed electroactivity by a remarkable high anodic current of $0.53 \pm 0.02$ mA cm$^{-2}$. The electroactivity of C. cochlearium is clearly linked to (planktonic) growth and glucose consumption. Thereby the secreted mediator or redoxactive center has a formal potential of $+0.22 \pm 0.05$ V vs Ag/AgCl sat. KCl, indicating that it is not a phenazine or vitamin. In contrast, In-silico analysis on candidate genes for DET showed that no strain under investigation was found to harbor as many genes as G. sulfurreducens or S. oneidensis for cytochrome containing proteins, but revealed that C. cochlearium has the genetic potential for nano-tube formation, while a flavin-based MET mechanism could be excluded.

Understanding EET and IET as an additional mode of interaction in microbial communities in general and in the gut specifically, will generate knowledge for both, human health and biotechnology. Although the role of EET is still to be revealed, one could speculate on vitamins, complex organic substance (i.e. fibers) [66], microorganisms or even the host as interaction partner, its importance becomes increasingly evident and may have a significant impact.

For instance, when considering deciphering electron transfer mechanisms within the mammalian gut one can dream of the design of prebiotics and drugs as well as an
improved diet. Therefore, an interplay of studies investigating microbial bioelectrochemistry, genetics as well as bioinformatics are necessary.

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7. References


[29] M.T. Madigan, K.S. Bender, D.H. Buckley, W.M. Sattley, D.A. Stahl, Brock


