Check for updates

ChemPubSoc Europe

DOI: 10.1002/celc.201901656

CHEMELECTROCHEM Articles

Clostridium Acetobutylicum's Connecting World: Cell Appendage Formation in Bioelectrochemical Systems

Mareike Engel,[a] André Gemünde,[a] Dirk Holtmann,[b] Christine Müller-Renno,[c] Christiane Ziegler,[c] Nils Tippkötter,[d] and Roland Ulber*[a]

Bacterial cell appendix formation supports cell-cell interaction, cell adhesion and cell movement. Additionally, in bioelectrochemical systems (BES), cell appendages have been shown to participate in extracellular electron transfer. In this work, the cell appendix formation of *Clostridium acetobutylicum* in biofilms of a BES are imaged and compared with conventional biofilms. Under all observed conditions, the cells possess filamentous appendages with a higher number and density in the BES. Differences in the amount of extracellular polymeric substance in the biofilms of the electrodes lead to the conclusion that the cathode can be used as electron donor and the anode as electron acceptor by *C. acetobutylicum*. When using conductive atomic force microscopy, a current response of about 15 nA is found for the cell appendages from the BES. This is the first report of conductivity for clostridial cell appendices and represents the basis for further studies on their role for biofilm formation and electron transfer.

1. Introduction

For bacterial cell-cell interactions as well as the connection of cells with surfaces for biofilm formation, cell appendices like flagella and pili play an important role during cell movement and adhesion.[1,2] Moreover, filamentous appendages can also carry out an extracellular electron transfer (EET) between microorganisms and electrodes in bioelectrochemical systems (BES). Two types of appendices enabling an EET have been identified and characterized so far: the conductive type-IV pili from *Geobacter sulfurreducens*[3] and membrane extrusions containing also periplasmatic components from *Shewanella oneidensis*.[4] Both types of appendices are commonly referred to as nanowires in the literature and vary in their diameter and their composition.[5,6] The conductive pili from *Geobacter* measure about 3–5 nm whereas for the membrane extrusions of *Shewanella* a wider range between 10–150 nm was found.[4,7,8] The EET by nanowires is generally considered as a direct extracellular electron transfer (DEET, also commonly abbreviated by DET in the literature) although newer results suggest that flavins that act as cofactors for *c*-type cytochromes on the surface of the appendices are probably responsible for the final electron transport step.[5,6,9]

For Gram-positive bacteria, a first hint for a DEET was found in 2009 by Marshall et al.[10] for the thermophilic bacterium *Thermincola ferriacetica* in a microbial fuel cell. The authors carried out cyclic voltammetry experiments and observed redox peaks for the cells in a biofilm on the electrode but no peaks appeared in the cell-free supernatant. It was therefore concluded that a DEET mechanism must be present and no soluble mediator was responsible for the electron transfer between the cells and the anode. Later Parameswaran et al.[11] analyzed an anodic biofilm of *T. ferriacetica* and found a dense network of cell appendages that they believed to be similar to *G. sulfurreducens* pili and that were thought to be conductive. However, only about half of the *c*-type cytochromes from *G. sulfurreducens* can be found in the genome of *T. ferriacetica*[12] and the conductivity has not been proven experimentally yet.

Choi et al.[13] reported in 2014 that the Gram-positive bacterium *Clostridium pasteurianum* was electroactive (without addition of an exogenous mediator) and that during cultivations at the cathode in a BES, higher butanol production could be achieved compared to conventional cultivations. Additionally, the authors observed the appearance of filamentous appendices of varying diameters in the biofilm on the cathode. In control cultivations, these appendages were not found. In the genome of *C. pasteurianum* no *c*-type cytochrome encoding genes can be found.[14] If the filamentous matrix does play a role in the electron transfer, it must thus be based on a mechanism other than the *c*-type cytochrome based EET described for the Gram-negative microorganisms of the genus *Geobacter* and *Shewanella*.[5,15,16] Recently, our group has demonstrated that an increased butanol production can also be achieved through a cathodic electro-fermentation in a BES with *C. acetobutylicum* compared to control cultivations.[17] Further

[a] *Dr. M. Engel, A. Gemünde, Prof. Dr. R. Ulber*
Bioprocess Engineering, University of Kaiserslautern
67663 Kaiserslautern, Germany
E-mail: ulber@mv.uni-kl.de
[b] *Prof. Dr. D. Holtmann*
Industrial Biotechnology, DECHEMA Research Institute
60486 Frankfurt am Main, Germany
[c] *Dr. C. Müller-Renno, Prof. Dr. C. Ziegler*
Department of Physics, University of Kaiserslautern
67663 Kaiserslautern, Germany
[d] *Prof. Dr. N. Tippkötter*
Bioprocess Engineering, University of Applied Science Aachen
52428 Jülich, Germany

Supporting information for this article is available on the WWW under https://doi.org/10.1002/celc.201901656

© 2019 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

21960216, 2020, 2, Downloaded from https://chemistry-europe.onlinelibrary.wiley.com/doi/10.1002/celc.201901656 by Rheinland-Pfälzische Technische Universität Kaiserslautern-Landau, Wiley Online Library on [03/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

studies indicate that flavins in mM concentrations are present in the supernatant of these electro-cultivations which might play a role for a mediated extracellular electron transport.[18] For *C. acetobutylicum* the only cell appendages which have been experimentally observed so far, are peritrichous flagella which assure the mobility of the cells.[19,20] In order to elucidate, whether *C. acetobutylicum* does also form cell appendages comparable to those of *C. pasteurianum* in a BES which might play a role for electron transfer, this work aims at visualizing planktonic cells and biofilms during cathodic electro-fermentations.

Experimental Section

Materials and Methods

Cultivation Conditions

Conventional cultivations were carried out in serum bottles using the strain *C. acetobutylicum* DSM 792 from the Leibnitz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). For pre-cultures the medium 104b recommended by the DSMZ and for the cultivation experiments a modified P2-medium (MP2opt) were prepared as described previously.[17] For biofilm analysis in conventional cultivations, a 2×2 cm^2 piece of a carbon fabric ACC-5092-15 (Kynol Europa GmbH, Hamburg, Germany) was added into the medium before autoclaving the serum bottles. Pre-culture cultivations were performed as described in[17].

Pre-Treatment of Electrode Materials

All working (WE) and counter electrodes (CE) were heated to 100 °C overnight for thermal desorption. Afterwards the electrodes were completely immersed in 2-propanol for at least 30 min and then the alcohol was washed off by three rinsing steps using DI-water and letting the electrode material rest in water for at least 30 min during each step. After washing, the electrodes were kept in DI-water until their use in an experiment.

Electro-Fermentations

Electro-fermentations were carried out either in an H-cell reactor (procedure and reactor described in[17]) or in a specially designed non-separated 1.3 L bioreactor using a rotating carbon brush (length 110 mm, diameter 21 mm, The Mill-Rose Company, Mentor, United States) as WE and a static carbon fabric from Kynol with a size of 13×28 cm^2 as CE (unpublished system). In order to achieve a rotating, conductive connection between the carbon brush and the potentiostat, a specially designed stainless steel shaft with a slip ring (B-command, Hamburg, Germany) was used. The rotational speed was fixed at the lowest speed (about 50 rpm) of a laboratory stirrer motor RW 20 (IKA-Werke GmbH, Staufen im Breisgau, Germany).

The WEs and CEs of both systems were prepared as described in section 2.2 and an Ag/AgCl electrode with saturated KCl as electrolyte (Sensortechnik Meinsberg, Meinsberg, Germany) was used as a reference. In both systems a potential of −600 mV against the reference electrode was applied at the WE. The cultivation conditions described in[17] were used.

Scanning Electron Microscopy

For scanning electron microscopy (SEM) imaging, planktonic cell samples and biofilms were washed twice with a 0.2 M sodium phosphate buffer pH 7.2, fixed in liquid nitrogen and afterwards freeze-dried in a Christ Alpha 2–4 freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Planktonic samples were placed on a 5 mm × 5 mm silicon support (Plano GmbH, Wetzlar, Germany). The freeze-dried samples were afterwards sputtered with a 3 nm iridium layer in a high vacuum coater Leica EM ACE600 (Leica Microsystems GmbH, Wetzlar, Germany). Imaging was carried out using a Hitachi SU8000 (Hitachi, Düsseldorf, Germany) and the corresponding software.

Flagella Staining

Flagella were visualized using a phase contrast microscope Eclipse Ni–U H550L (Nikon GmbH, Duesseldorf, Germany) after staining with a method adopted from Blenden and Goldberg[21]: A bacterial sample was taken from a culture, centrifuged and resuspended in water. 8 μL of this cell suspension were put onto a clean microscopic slide and left at room temperature for drying. Afterwards 30 μL of a solution consisting of 1 g tannic acid, 0.3 g $FeCl_3$, 0.4 L 15 % formaldehyde and 0.1 mL 0.5 M NaOH in 100 L DI-water were added. After 4 min incubation at room temperature the microscopic slide was washed with DI-water. A second solution was prepared by dissolving 0.02 g silver nitrate in 1 mL DI-water and adding dropwise a few microliters of a 25 % ammonia solution until a brown precipitate of silver oxide appeared and vanished again during formation of $[Ag(NH_3)_2]^+$. Afterwards, more silver nitrate was added until a pH of about 10 was reached visible by an increase in turbidity of the solution. The sample was covered for 30 s with this solution before another washing step was performed. Prior to the microscopic imaging the sample on the slide was completely let dry at room temperature.

Conductive Atomic Force Microscopy

Samples for conductive atomic force microscopy (cAFM) were taken from a WE compartment of an H-cell cultivation. Samples were filtered using a 0.22 μm nylon syringe filter and the cells were afterwards washed using a 0.9 % NaCl solution. Then the biomass was resuspended in ultrapure water, fixed with 2.5 % glutaraldehyde and subsequently dehydrated in ethanol solutions (30 %, 50 %, 70 %,90 %, 100 %). The last ethanol step was used twice and finally about 5 μL were placed on a freshly cleaved piece of highly oriented pyrolytic graphite (HOPG) with a mosaicity of $0.8° \pm 0.2°$ (Plano GmbH, Wetzlar, Germany) and air-dried. cAFM measurements were carried out in the *QI*™-modus on a NanoWizard 3 (JPK Instruments AG, Berlin, Germany) and the corresponding software JPKSPM Data Processing. A platinum coated cantilever ElectriCont-G (Budget Sensors, Sofia, Bulgaria) with a spring constant of 0.2 N m^{-1} was used and a voltage of 1 V was applied between the tip and the sample holder.

2. Results and Discussion

2.1. Cell Appendage Formation During Electro-Fermentations

SEM images of biofilms from the WE and the CE of the specially designed bioelectrochemical reactor containing the WE and CE in one compartment were taken (see section 2.3). At the WE (cathode) a potential of −600 mV vs. Ag/AgCl was applied.

© 2019 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA

21960216, 2020, 2, Downloaded from https://chemistry-europe.onlinelibrary.wiley.com/doi/10.1002/celc.201901656 by Rheinland-Pfälzische Technische Universität Kaiserslautern-Landau, Wiley Online Library on [03/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

During the electro-cultivation, the formation of a very thick biofilm on the WE was observed (see Figure 1A). Biofilm formation at the CE also took place but was more heterogeneous than at the cathode (see Figure 1B). Figure 2A shows the micrograph of the dense biofilm on the WE with the enlarged part in Figure 2B illustrating typical filamentous connections found. Additionally, on the WE, a mesh-like structure was observed at various spots of the sample (see Figure 2C). When imaging an individual cell as in Figure 2D, it was visible that several appendices originated from a single cell with various shapes ranging from very straight filaments (e.g. red arrow) to more curvy ones (e.g. blue arrow). The latter correspond in their shape and in their dimensions with about 20 nm in diameter and 10–20 μm in length to the expected flagella.[20,22] For the other type of appendages, it cannot be concluded only by the SEM images, whether these appendices are also flagella appearing differently or being coated with extracellular polymeric substance (EPS) or cell debris material or if these appendages represent a new class of appendices not yet identified for *Clostridia.*

Figure 1. *C. acetobutylicum* biofilm formed on (A) the WE (length of the carbon brush: 110 mm, diameter of the brush: 21 mm) and (B) the CE after 48 h of an electro-fermentation at −600 mV in a single chamber bioelectrochemical reactor.

The observed structures, including the mesh-like filaments or very straight and long structures resemble in their appearance the conductive nanowires found for *S. oneidensis.*[4,8,23,24] Dohnalkova et al.[25] analyzed *S. oneidensis* biofilms using various microscopic techniques. They found that the EPS appeared as a closed film when they were using a cryo-SEM, but with conventional SEM imaging, the biofilm did show individual cells and various appendices as was found in this work for *C. acetobutylicum.* Additionally, they confirmed that the filamentous appendages were not only a product of the sample preparation for SEM imaging, since they observed appendix formation for living cells in an aqueous environment in real-time.[25] Moreover, Karcz et al.[26] found that when using environmental scanning electron microscopy for imaging a consortium of bacteria, only the hydrated EPS was visible. But after cryofixation and conventional SEM imaging, a mesh-like structure comparable to Figure 2C was observed. From these studies, it can be concluded that the structures found in this work are probably consistent of cell appendices and EPS. When comparing the SEM images with the nanowires in the literature

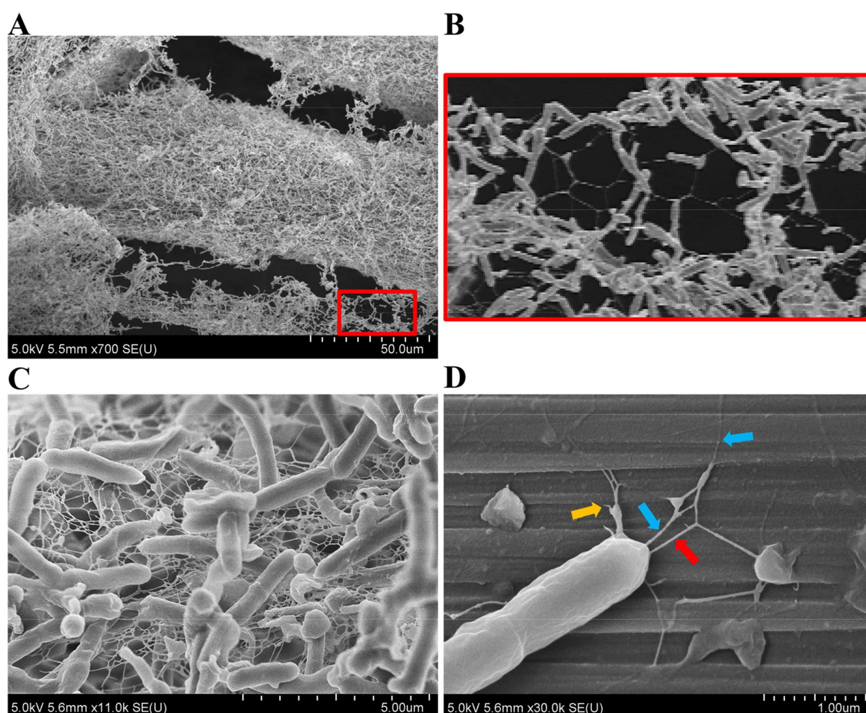

Figure 2. SEM micrographs of a *C. acetobutylicum* biofilm on the WE of an electro-fermentation. (A) Overview of the biofilm structure. (B) Enlarged part from (A) showing a broad mesh-like structure. (C) Dense mesh-like structure. (D) Individual cell with various filaments. Red arrow indicates a straight appendage, blue arrow represents possibly a flagellum and yellow arrow shows a more irregular filament.

 © 2019 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA

of *S. oneidensis*, there is a high resemblance of the *Shewanella* membrane extrusions and the observed appendages from *C. acetobutylicum*.[8,27,28]

Since biofilm formation was also observed on the anode (CE) in the bioelectrochemical reactor, a sample from the CE was imaged (see Figure 3). Once more a dense network of filaments was observed. The cells in these images seem to be less covered by EPS in comparison to the micrographs of the cathodic sample. Additionally, on the CE, the observed network contains fewer agglomerates compared to the biofilm of the WE (see Figure 3A). Nevertheless, the cells are also connected by filamentous appendages which mainly originate from the cell poles as has been asserted at the WE (see Figure 3B). A mesh-like filament like on the WE (Figure 2C) was not found for the biofilm of the CE.

Since biofilm formation occurred on both electrodes, it is likely that the cells are able to use an electrode as electron donor as well as acceptor. This hypothesis is supported by the differences in EPS amounts in the two biofilms. EPS contains polysaccharides which can be reused by the cells as carbon and electron supply in case of an electron limitation.[29] If the electrode is used as electron acceptor by the bacteria, EPS could

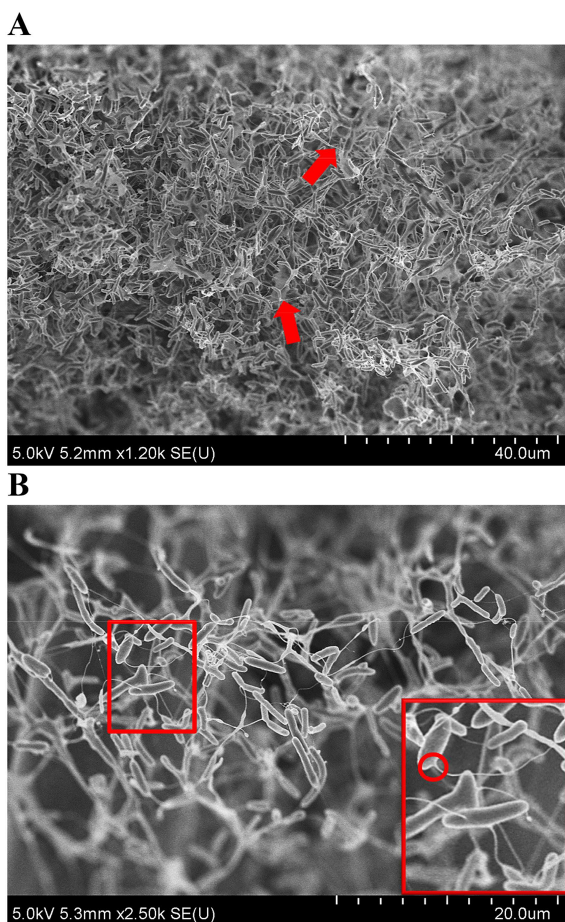

Figure 3. SEM micrographs of a biofilm of *C acetobutylicum* on the CE after 48 h electro-fermentation. (A) Overview of the biofilm structure with red arrows indicating examples for filamentous connections between cells. (B) Higher magnification of the network in the biofilm. In the red frame the red circle highlights the origin of one of the filaments on a cell.

thus be hydrolyzed to act as additional electron donor for the cells. On the other hand, an electrode which acts as electron donor for the cells will not only avoid an electron limitation but might even create an electron excess. The formation of EPS can then be a way for the cells to get rid of these excess electrons, leading to a biofilm with high EPS content. These differences in EPS formation between the electron donating electrode (cathode) and an electron accepting electrode (anode) have been observed in this study indicating that the cells interact with both electrodes. The biofilm on the WE (cathode, electron donor) uses the electrode as additional electron donor and consequently a high amount of EPS is visible in Figure 1A and Figure 2. The biofilm on the CE (anode, electron acceptor) in Figure 1B and Figure 3, in contrast, only contains low amounts of EPS leading to a "clean" appearance of the cells and the filaments. In this biofilm, the cells probably donate their electrons to the anode and hydrolyze the EPS as additional electron source. This connection of the electrode acting as electron donor or acceptor for the bacterial cells and the EPS formation or hydrolyzation should be studied further in the future.

Overall, the results from the bioelectrochemical reactor show that cell appendage formation takes place at the WE as well as the CE. In the following a biofilm from a non-electrochemical biofilm was imaged as control to compare the observed cell appendices during electro-fermentation with those in conventional cultivations.

2.2. Cell Appendage Formation in Conventional Cultivations

Figure 4 illustrates examples of filamentous networks found in a conventional biofilm grown on a carbon fabric in a serum bottle. As can be seen from Figure 4A, there are thick clusters containing cells and EPS that are connected to individual cells or other agglomerates by the filaments. An example of a straight filament measuring more than 20 μm in length and containing a cell (red circle) is shown in Figure 4B. Most of these filaments seem to originate close to the cell poles. However, the cell highlighted with the red arrow is an example of a cell containing several appendices starting from various locations of the cell surface. In Figure 4C, it is shown that at some places the appendages form a mesh-like structure and Figure 4D illustrates the expected flagella with their wavy form and their typical dimensions of about 20 nm in diameter and a length of 16–22 μm.[20] When a flagella staining was carried out for planktonic cells of this culture (see supplementary Figure S1). A very high number of flagella was found. In the SEM images of the biofilm, less flagella were observed. Those flagella were mostly located in close vicinity to the cells on the carbon fabric's surface and not in direct contact with the cells in the 3D-matrix. Since a cryofixation was used during sample preparation, it is likely that the flagella were cleaved off during the freezing process. Thicker appendices however remained attached to the cells.

The sample for the micrographs in Figure 4 had been taken from a non-electrochemical culture. Nevertheless, appendices which possess a different appearance in their shape and

© 2019 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA

21960216, 2020, 2, Downloaded from https://chemistry-europe.onlinelibrary.wiley.com/doi/10.1002/celc.201901656 by Rheinland-Pfälzische Technische Universität Kaiserslautern-Landau, Wiley Online Library on [03/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

21960216, 2020, 2, Downloaded from https://chemistry-europe.onlinelibrary.wiley.com/doi/10.1002/celc.201901656 by Rheinland-Pfälzische Technische Universität Kaiserslautern-Landau, Wiley Online Library on [03/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

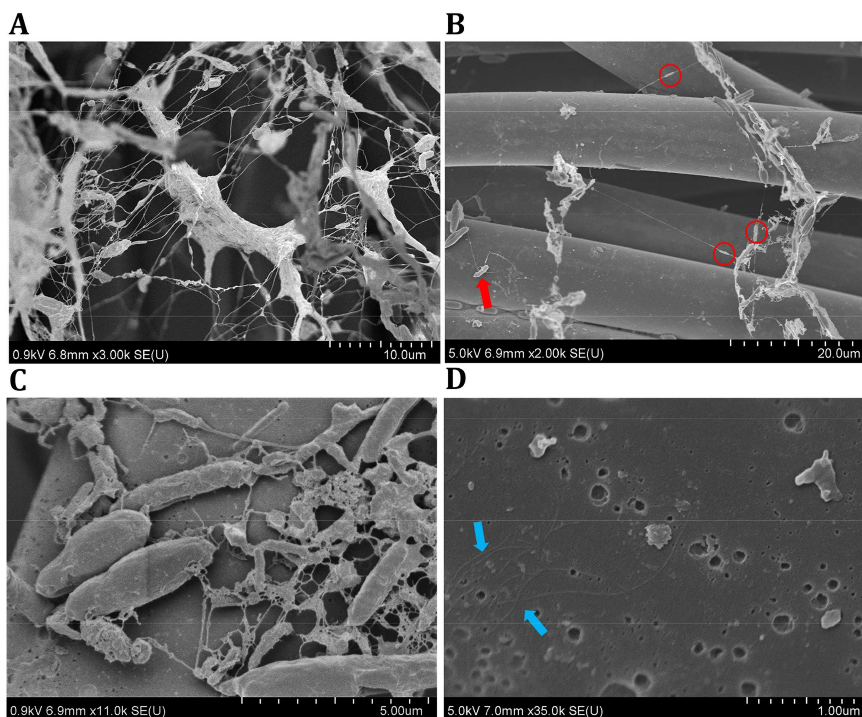

Figure 4. SEM micrographs of a biofilm of *C. acetobutylicum* grown on a carbon fabric surface immersed in medium of a serum bottle cultivation after 72 h (non-electrochemical system). (A) Network with clusters containing cells and EPS. (B) Individual cells (red circles) connected with long filamentous structures. (C) Cells connected by a mesh-like filamentous structure. (D) Detached flagella on the surface of the carbon fabric.

diameter from the expected flagella were found. The observed appendices in the *C. acetobutylicum* biofilm vary in their diameter in a range of 15 μm to more than 100 μm which is consistent with the dimensions of cell appendices found for *C. pasteurianum*[13] in electro-fermentations and the cell appendices found in the electrochemical biofilms presented above (see Figure 2 and Figure 3).

For *C. acetobutylicum,* it was previously shown that in a purely synthetic medium, cells tended to stick together and to form agglomerates and biofilms.[17] When a complex compound like yeast extract was added, the cell suspension did not contain any bigger clusters. It was suggested that the biofilm and cluster formation was due to a certain limitation present in the purely synthetic medium which can be circumvented by adding yeast extract. The SEM images in this work represent a culture from the same synthetic medium as used in[17] and thus confirm that the cells connect to one another to form a network in this medium. This network probably allows a cell-cell interaction for nutrient transport or nutrient entrapment which has been reported for low nutrient environments for other species.[30] Choi et al.[13] also used a purely synthetic medium whose composition is close to the composition of the medium used in this study during cultivation of *C. pasteurianum*. In contrast to their work however, our experiments indicate that cell appendages are not exclusively formed when an electric potential is applied.

2.3. Conductivity of Cell Appendices of *C. Acetobutylicum*

As shown above, the formation of cell appendages is not limited to the BES. However, in the BES, filament density was higher and longer distances were covered compared to the control cultivation in the serum bottle. In order to elucidate whether the observed filamentous appendices are conductive, cAFM measurements were performed. For the membrane extrusions from *Shewanella* as well as for the pili from *Geobacter* this cAFM measurements have been successfully applied after chemical fixation (and sequential dehydration in ethanol) to demonstrate that the nanowires induce a current response when a potential is applied.[16,23,27,31] In order to obtain comparable conditions, a sample from the cathodic compartment of an electro-cultivation in a separated H-cell reactor was chemically fixed, dehydrated and visualized according to previously published methods.[16,23,27,31]

Figure 5A illustrates the pole of an individual cell (left bottom corner) with two possible appendices (blue and green arrow). The gray arrows indicate steps in the HOPG surface which are distinguishable from the putative cell appendages from their straight appearance. In Figure 5B, the corresponding current response when a voltage of 1 V was applied is illustrated. The diameter of the wavy appendix highlighted with the blue arrow was determined as 19 nm. Due to its appearance and size, it is most likely a flagellum. The other putative appendage (green arrow) appears in a straight form as has been observed for some filaments in the SEM images. These two types of appendages match thus the observations during

 © 2019 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA

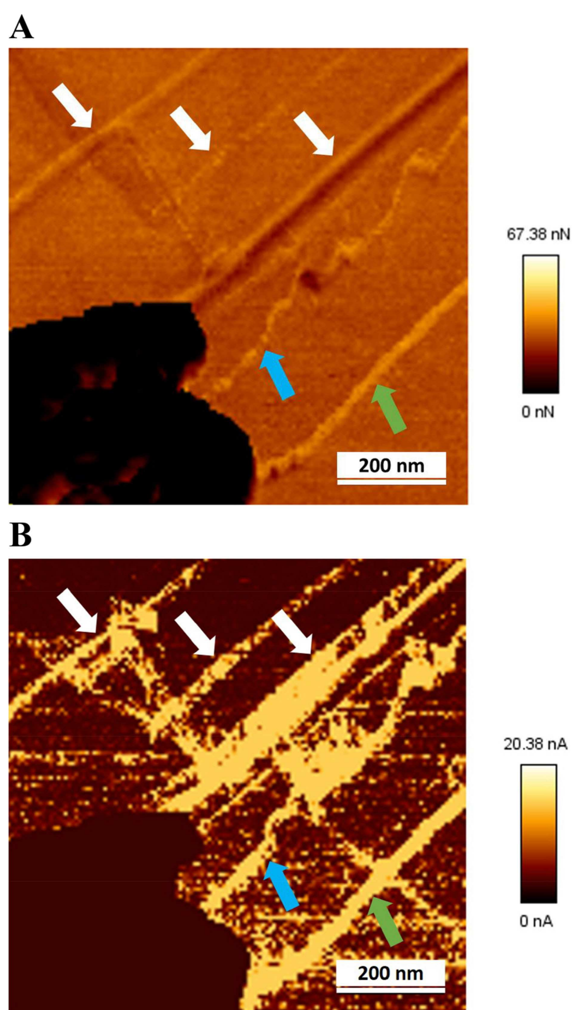

Figure 5. (A) Adhesion and (B) current response of cAFM measurements at 1 V of *C. acetobutylicum* cell from a planktonic culture cultivated at −600 mV in an H-cell BES.

SEM analysis. Moreover, in agreement with the SEM images, both types of appendages originate from the cell pole.

When considering Figure 5B, it can be concluded that the cell acts as an insulator since no current response was measured at the cell surface. The hypothesized appendices however as well as the steps in the HOPG material resulted in a current response of about 15 nA. For the type IV-pili from *G. sulfurreducens,* a current response of 6 nA was measured for an applied voltage of 600 mV.[31] For the membrane extrusions from *S. oneidensis* MR-1, a similar current of 6 nA was observed when 800 mV were applied. The current response shown by the *C. acetobutylicum* cell appendages are higher which is probably due to the higher voltage of 1 V applied in this study. The observed current response of the steps of the HOPG sample (straight lines) has been studied previously and is due to the mechanical pretreatment of the substrate or the crystal structure.[32]

To the author's best knowledge, this work represents the first report of a current response of cell appendices of a Gram-positive bacterium. It has been suggested that Gram-positive bacteria also possess type-IV pili comparable to those of *Geobacter* species.[33] The *C. acetobutylicum*'s genome indeed shows a gene cluster for expression of proteins for type-IV pili synthesis and secretion (Cac_2102-Cac_2105, Cac_1980 and Cac_1690).[14,34] However, the observed appendices in this study are unlikely to represent pili, since the diameters found with more than 20 μm do not match the very thin pili with less than 10 μm.[2,35] The size and morphology of the appendages in this study resemble the membrane extrusions from *S. oneidensis*. For these membrane extrusions, it was shown that *c*-type cytochromes were responsible for the electron transfer.[16,31] Since there is only one hint for a *c*-type cytochrome-like protein in the genome of *C. acetobutylicum* (Cac_2528),[14] there has to be an alternative mechanism for electron transport in this organism. Whether flagella can be conductive has not yet been identified conclusively.[20,22,36] It has been shown that the bacterium *Pelotomaculum thermopropionicum* which belongs to the genus *Clostridia* transfers electrons in the form of hydrogen to cells of *Methanothermobacter thermoautotrophicus* by filamentous connections which have been demonstrated to mainly contain flagellin.[37] Those flagellin containing appendages appeared either in a wavy, flagella like form or as a straight filament. Therefore, both the curvy as well as the straight filaments observed in this work might be all based on flagella. This should be studied further by using for example fluorescent staining techniques in the future.

Furthermore, as discussed in section 3.1, the appendices/flagella are probably covered at least partially by EPS. Consequently, it is possible that the observed conductivity is produced by EPS components and not by the filament itself. EPS contains, besides proteins, other conductive compounds like DNA or humic substances and was proven to be conductive for *S. oneidensis, B. subtilis* and the yeast *Pichia pistoris.*[25,38] All those electroactive EPS structures that were studied contained flavins. It was shown recently that during electro-fermentation with *C. acetobutylicum* flavin secretion was increased.[18] The EPS structure supplies a matrix that allows the bacterium to maintain the secreted flavins close to the cells and consequently close to the electrode. This would facilitate the electron transfer between the electrode and the cells. Whether flavin molecules are responsible for the observed current response in the cAFM measurements or whether the conductivity is based on another mechanism still needs further elucidations. For *G. sulfurreducens*, it was shown by Rollefson et al.[39] that mutants that did produce pili and *c*-type cytochromes but lacked the ability to excrete polysaccharides for EPS formation were not able to carry out an EET to iron(III)-particles. Moreover, those mutants detached from an electrode, once a potential was applied. The authors concluded that the EPS is necessary for the electron transport by binding *c*-type cytochromes and maybe other substances that allow the conductivity despite the formation of conductive appendices. *Geobacter* however forms thinner biofilms than *C. acetobutylicum* and its pili are thought to be responsible for formation of biofilms >10 μm.[40] It is therefore possible that the thick biofilms observed for *Clostridia* are at least partly possible because of the cell appendages but that the conductivity is assured by redox active compounds

© 2019 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA

21960216, 2020, 2, Downloaded from https://chemistry-europe.onlinelibrary.wiley.com/doi/10.1002/celc.201901656 by Rheinland-Pfälzische Technische Universität Kaiserslautern-Landau, Wiley Online Library on [03/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

21960216, 2020, 2, Downloaded from https://chemistry-europe.onlinelibrary.wiley.com/doi/10.1002/celc.201901656 by Rheinland-Pfälzische Technische Universität Kaiserslautern-Landau, Wiley Online Library on [03/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

that are retained within the EPS matrix. After sample preparation for AFM imaging the EPS components will be found on the appendage's surface which could explain the observed conductivity through the measured current response. The conductivity of the EPS should be studied further by separating the matrix from the cells and analyzing the conductive properties by electrochemical techniques.

3. Conclusions

In this work, it was shown for the first time that the Gram-positive bacterium *C. acetobutylicum* creates a network of appendices connecting the cells to one another and to an electrode surface during conventional cultivation and in a BES when an electric potential is applied. In contrast to previous work on *C. pasteurianum*,[13] our experiments, however, clearly show that the appendage formation for *C. acetobutylicum* is not limited to the BES but also takes place during conventional cultivation. Additionally, it was found that for appendages formed in the BES during electro-fermentation a current response of 15 nA could be measured when a potential of 1 V was applied during cAFM measurements. The observed appendages were on the one hand identified as flagella and on the other hand, thicker filaments whose nature still needs to be identified but which might be flagella coated by EPS were found. Both types of filaments showed identical conductivity. The results are very promising to conduct an in-depth study of the conductivity of clostridial biofilms and their electron transport mechanisms in bioelectrochemical systems in the future.

Acknowledgements

The authors acknowledge funding by the German Federal Environmental Organization "Deutsche Bundesstiftung Umwelt" (DBU PhD scholarship grant 20015/367) and the Deutsche Forschungsgemeinschaft in the frame of the Collaborative Research Center CRC 926 "Microscale Morphology of Component Surfaces".

Conflict of Interest

The authors declare no conflict of interest.

Keywords: cell adhesion · Clostridia · electron transfer · microbial electrochemistry · nanowires

[1] J. Haiko, B. Westerlund-Wikström, *Biology* **2013**, *2*, 1242–1267.
[2] T. A. Smirnova, L. V. Didenko, R. R. Azizbekyan, Y. M. Romanova, *Microbiology* **2010**, *79*, 413–423.
[3] G. Reguera, *Trends Microbiol.* **2011**, *19*, 105–113.
[4] S. Pirbadian, S. E. Barchinger, K. M. Leung, H. S. Byun, Y. Jangir, R. A. Bouhenni, S. B. Reed, M. F. Romine, D. A. Saffarini, L. Shi, Y. A. Gorby, J. H. Golbeck, M. Y. El-Naggar, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 12883–12888.
[5] T. Boesen, L. P. Nielsen, *mBio* **2013**, *4*, e00270-13.
[6] M. Y. El-Naggar, Y. A. Gorby, W. Xia, K. H. Nealson, *Biophys. J.* **2008**, *95*, L10-2.
[7] D. R. Lovley, K. P. Nevin, *Curr. Opin. Biotechnol.* **2011**, *22*, 441–448.
[8] Y. A. Gorby, S. Yanina, J. S. McLean, K. M. Rosso, D. Moyles, A. C. Dohnalkova, T. J. Beveridge, I. S. Chang, B.-H. Kim, K. S. Kim, D. E. Culley, S. B. Reed, M. F. Romine, D. A. Saffarini, E. A. Hill, L. Shi, D. A. Elias, D. W. Kennedy, G. Pinchuk, K. Watanabe, S. Ishii, B. Logan, K. H. Nealson, J. K. Fredrickson, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11358–11363.
[9] A. Okamoto, R. Nakamura, K. H. Nealson, K. Hashimoto, *ChemElectroChem* **2014**, *1*, 1808–1812.
[10] C. W. Marshall, H. D. May, *Energy Environ. Sci.* **2009**, *2*, 699.
[11] P. Parameswaran, T. Bry, S. C. Popat, B. G. Lusk, B. E. Rittmann, C. I. Torres, *Environ. Sci. Technol.* **2013**, *47*, 4934–4940.
[12] B. G. Lusk, J. P. Badalamenti, P. Parameswaran, D. R. Bond, C. I. Torres, *Genome Announc* **2015**, *3*.
[13] O. Choi, T. Kim, H. M. Woo, Y. Um, *Sci. Rep.* **2014**, *4*, 1–10.
[14] M. Kanehisa, "Kyoto Encyclopedia of Genes and Genomes", can be found under http://www.kegg.jp/, **2017**.
[15] D. R. Bond, S. M. Strycharz-Glaven, L. M. Tender, C. I. Torres, *ChemSusChem* **2012**, *5*, 1099–1105.
[16] M. Y. El-Naggar, G. Wanger, K. M. Leung, T. D. Yuzvinsky, G. Southam, J. Yang, W. M. Lau, K. H. Nealson, Y. A. Gorby, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 18127–18131.
[17] M. Engel, D. Holtmann, R. Ulber, N. Tippkötter, *Biotechnol. J.* **2019**, *14*, e1800514.
[18] M. Engel, H. Bayer, D. Holtmann, N. Tippkötter, R. Ulber, *Bioelectrochemistry* **2019**, *129*, 242–250.
[19] W. B. Whitman, *Bergey's Manual of Systematic Bacteriology. Volume Three The Firmicutes*, Springer-Verlag New York, New York, **2009**.
[20] N. A. Gutierrez, *Dissertation*, Massey University, Palmerston North (Neuseeland), **1989**.
[21] D. C. Blenden, H. S. Goldberg, *J. Bacteriol.* **1965**, *89*, 899–900.
[22] R. Macnab, *J. Bacteriol.* **1999**, *181*, 7149–7153.
[23] K. M. Leung, *Dissertation*, University of Western Ontario, London (Canada), **2011**.
[24] O. Bretschger, A. Obraztsova, C. A. Sturm, I. S. Chang, Y. A. Gorby, S. B. Reed, D. E. Culley, C. L. Reardon, S. Barua, M. F. Romine, J. Zhou, A. S. Beliaev, R. Bouhenni, D. Saffarini, F. Mansfeld, B. H. Kim, J. K. Fredrickson, K. H. Nealson, *Appl. Environ. Microbiol.* **2007**, *73*, 7003–7012.
[25] A. C. Dohnalkova, M. J. Marshall, B. W. Arey, K. H. Williams, E. C. Buck, J. K. Fredrickson, *Appl. Environ. Microbiol.* **2011**, *77*, 1254–1262.
[26] J. Karcz, T. Bernas, A. Nowak, E. Talik, A. Woznica, *Scanning* **2012**, *34*, 26–36.
[27] K. M. Leung, G. Wanger, M. Y. El-Naggar, Y. A. Gorby, G. Southam, W. M. Lau, J. Yang, *Nano Lett.* **2013**, *13*, 2407–2411.
[28] Y. Chao, T. Zhang, *Appl. Microbiol. Biotechnol.* **2011**, *92*, 381–392.
[29] C. S. Laspidou, B. E. Rittmann, *Water Res.* **2002**, *36*, 1983–1992.
[30] a) R. F. Müller, *Water Res.* **1996**, *30*, 2681–2690; b) J. W. Costerton, Z. Lewandowski, D. E. Caldwell, D. R. Korber, H. M. Lappin-Scott, *Annu. Rev. Microbiol.* **1995**, *49*, 711–745.
[31] G. Reguera, K. D. McCarthy, T. Mehta, J. S. Nicoll, M. T. Tuominen, D. R. Lovley, *Nature* **2005**, *435*, 1098–1101.
[32] S. Banerjee, M. Sardar, N. Gayathri, A. K. Tyagi, B. Raj, *Phys. Rev. B* **2005**, *72*, 565.
[33] S. Melville, L. Craig, *Microbiol. Mol. Biol. Rev.* **2013**, *77*, 323–341.
[34] J. Zhou, K. E. Rudd, *Nucleic Acids Res.* **2013**, *41*, D613-24.
[35] A.-B. Jonson, S. Normark, M. Rhen, *Contrib. Microbiol.* **2005**, *12*, 67–89.
[36] S. K. Sure, L. M. Ackland, A. A. Torriero, A. Adholeya, M. Kochar, *Microbiology* **2016**.
[37] a) S.'i. Ishii, T. Kosaka, K. Hori, Y. Hotta, K. Watanabe, *Appl. Environ. Microbiol.* **2005**, *71*, 7838–7845; b) T. Shimoyama, S. Kato, S.'i. Ishii, K. Watanabe, *Science* **2009**, *323*, 1574.
[38] Y. Xiao, E. Zhang, J. Zhang, Y. Dai, Z. Yang, H. E. M. Christensen, J. Ulstrup, F. Zhao, *Sci. Adv.* **2017**, *3*, e1700623.
[39] J. B. Rollefson, C. S. Stephen, M. Tien, D. R. Bond, *J. Bacteriol.* **2011**, *193*, 1023–1033.
[40] a) R. J. Steidl, S. Lampa-Pastirk, G. Reguera, *Nat. Commun.* **2016**, *7*, 1–6; b) R. S. Renslow, J. T. Babauta, A. Dohnalkova, M. Boyanov, K. M. Kemner, P. D. Majors, J. K. Fredrickson, H. Beyenal, *Energy Environ. Sci.* **2013**, *6*, 1827–1836.

Manuscript received: October 3, 2019
Revised manuscript received: November 11, 2019
Accepted manuscript online: November 14, 2019

 © 2019 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA