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Metabolic engineering of a novel strain of electrogenic bacterium *Arcobacter butzleri* to create a platform for single analyte detection using a microbial fuel cell

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ABSTRACT

Electrogenic bacteria metabolize organic substrates by transferring electrons to the external electrode, with subsequent electricity generation. In this proof-of-concept study, we present a novel strain of a known, electrogenic *Arcobacter butzleri* that can grow primarily on acetate and lactate and its electric current density is positively correlated (R^2 = 0.95) to the COD concentrations up to 200 ppm. Using CRISPR-Cas9 and Cpf1, we engineered knockout *Arcobacter butzleri* mutants in either the acetate or lactate metabolic pathway, limiting their energy metabolism to a single carbon source. After genome editing, the expression of either acetate kinase, *ackA*, or lactate permease, *lctP*, was inhibited, as indicated by qPCR results. All mutants retain electrogenic activity when inoculated into a microbial fuel cell, yielding average current densities of 81–82 mA/m^2, with wild type controls reaching 85–87 mA^2. In the case of mutants, however, current is only generated in the presence of the substrate for the remaining pathway. Thus, we demonstrate that it is possible to obtain electric signal corresponding to the specific organic compound via genome editing. The outcome of this study also indicates that the application of electrogenic bacteria can be expanded by genome engineering.

1. Introduction

Common in various environments, including wastewater [1], anaerobic sludge [2], surface water [3], ground water [4] and seawater [5], *Arcobacter* spp. belong to the class of Epsilonproteobacteria and comprises 27 species and 39 strains, with 13 potentially new species, with 4 distinct clusters [6]. Among them, some of the species have been found to be Mn and Fe reducers [7], which indicates that they can be electrogenic. Electrogenicity is an ability of certain microorganisms to donate eletrons to solid electron acceptor upon anaerobic respiration [8]. Electrons from the cell can be passed either through outer membrane multiheme cytochromes [9], conductive appendages (e-pili) [10] or via extracellular, soluble redox mediators, e.g. quinones and flavins [11]. Such a process, termed extracellular electron transfer (EET) can be harnessed in a form of electricity-producing fuel cell, hence termed Microbial Fuel Cell (MFC) [12]. In an MFC, organic matter, such as wastewater, serves as electron donor and anode, populated by electrochemically-active bacteria (EAB) [13,8], is a terminal electron acceptor. In most common MFCs, EET is then coupled with either chemical or biological reduction of oxygen at the cathode to allow electricity generation [14]. Indeed, *A. butzleri* ED-1 has been previously isolated from the anode of a acetate-fed MFC [15], therefore can be termed as EAB. It has also been found in other MFC communities [16–18], indicating it is a versatile, but not dominant, EAB. Although some strains of *A. butzleri* are associated with diseases in animals [19] and have also been found in human diarrhoea samples [20,21], they seem to be not initial pathogenic agents in humans, but rather, opportunistic organisms growing in immunocompromised patients [22].

Further studies have shown that *A. butzleri* ED-1 is only able to grow on acetate or lactate, with moderate growth on succinate [15]. Fully annotated genomes of *A. butzleri* ED-1 and the genetically similar strain L have been obtained and annotated [23]. It has also been demonstrated that *A.butzleri* RM048 can be genetically modified, using electroporation [24].

Clustered, regularly interspaced, palindromic repeats (CRISPR) is a prokaryotic immune system that protects microbes against foreign

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genetic elements [25,26]. It can recognize and cleave specific sequences and has become a powerful tool in biotechnology, allowing precise genome editing [27,28], even at multiple loci simultaneously [29]. Several CRISPR-assisted nuclease enzymes have been utilized for genome editing, the most popular being Cas9 [27] and Cas12a(Cpf1) [30],which introduce blunt and staggered DNA cuts, respectively. Being native to 50% of sequenced bacteria, CRISPR may sometimes cause interference to the host cell [31]. In the case of *A. butzleri*, strain L contains a CRISPR region in the genome [23]; therefore, it may have its own endonuclease system. Strain ED-1, however, does not have such region and genus-wide study revealed that CRISPR-associated genes are found in 47% of *Arcobacter* genomes [6]. Although most vectors for CRISPR systems are developed for eukaryotes, vectors for bacteria are also available [32–35] some work has demonstrated that it is possible to recombine e.g. cyanobacteria [36] or gamma-proteobacterium *Shewanella oneidensis* [37] with Cas9, whereas Cpf1 has been applied to engineer gram-positive *Corynebacterium glutamicum* [38], but not in Proteobacteria. In the aforementioned studies, CRISPR-mediated editing has been conducted via plasmid integration. It has been demonstrated, however, that the use of plasmid may increase the off-target effects, induce cell toxicity and plasmid may integrate into host genome [39,40]. With the advances in the CRISPR technology, ribonucleoprotein (RNP) complex delivery has been demonstrated, which can reduce off-target effects, and increases efficiency, especially for knock-in mutations, due to lesser degradation probability [41–44]. Moreover, no functional assays were performed on EAB after CRISPR-mediated genome editing. As EAB often utilize multiple carbon (fuel) sources whilst using complex metabolic pathways for electron transfer and subsequent current generation, we became interested in the application of *A. butzleri* to detect specific organic substrates. This is especially important in the bioethanol industry, where the detection of low concentrations of inhibitory by-products, e.g. acetate and lactate, is crucial for maintaining high yields of ethanol [45]. Detecting acetate and lactate is time-consuming and expensive. It also requires the use of offline techniques, such as ion, gas or liquid chromatography [46]. Moreover, most available live biosensors are unable to distinguish easily between acetate and lactate. In this study, we successfully delivered CRISPR Cas9 and Cpf1 RNPs via RNP into a novel *A. butzleri* strain to delete either acetate or lactate metabolism. We also showed that the edited mutants are still capable of extracellular electron transfer, although only when grown on the carbon source for which a functional pathway remained.

2. Materials and methods

2.1. Isolation

Sediment from Okinawa mangrove forests (100 mL) was collected from the aerobic/anaerobic zone (50 cm) and diluted 1:3 with the basal medium (as described in [15]), containing 200 mg/L $CaCl_2 \cdot 2H_2O$, 250 mg/L $MgCl_2 \cdot 6H_2O$, 500 mg/L NH_4Cl and transferred into a 0.5 L bottle with a 3-electrode system: a working electrode (carbon cloth), a counter electrode (graphite rod) and a reference electrode (Ag/AgCl), and filled with the basal medium with 1 g/L with acetate (COD). Using a potentiostat (UniChem), −0.25 V (vs. Ag/AgCl) was applied to working electrode. After 7 days, the solution was replaced with basal medium with 1 g/L COD. The bottle was left at open circuit mode and the anode potential was monitored. Once anode potential became negative, which indicated the presence of metabolically-active EAB, it was connected for another 7 days at −0.25 V. Samples from each collection were subjected to DNA extraction and PCR was performed with *A. butzleri* using ED-1 specific 16S rRNA primers (Table S1). Subsequently, the same samples were incubated in 5 mL Arcobacter Broth (Oxoid, CM0965), supplemented with pyruvate-containing Campylobacter blood-free selective agar base (CM0739) with addition of antibiotics: cefoperazone (16 ug/ml), novobiocin (32 ug/ml), and trimethoprim (64 ug/ml), as described elsewhere [15]. 10 uL from the positive collections were then plated on Arcobacter Broth agar and incubated microaerobically (5% O_2, 10% CO_2) in a gas jar with a microaerophilic gas sachet (Mitsubishi Chemical Group) at 30 °C for 12 h. Growth was tested using basal medium with addition of 1 g/L one of the following carbon source: acetate, lactate or succinate. Single colonies were picked, and incubated in 10 mL Arcobacter Broth with antibiotics, until OD 0.8. Stock cultures were made with 30% v/v glycerol and DNA was extracted using the Maxwell RSC GMO purefood protocol (Promega). Samples for microscopic imaging were taken simultaneously with the DNA samples and processed with osmium, as follows: upon removal from the anode compartment, the samples was immediately cut by knife, and fixed by 1 % Osmium diluted with 0.2 M Cacodylate (Wako) buffer 30 min. The samples were then washed three times with MiliQ water and dehydrated stepwise with a graded series of ethanol solutions (70, 80, 90, 95 and three times 100%). The electrode samples were finally critical-point dried with tert-butyl ethanol and sputter coated with a thin layer of gold. The samples were analyzed by a scanning electron microscopy (SEM)(JSM-7900F JEOL).

2.2. Genome sequencing and annotation

Single clones were subjected to HiSeq and PacBio cloning. Reads were mapped to the reference genome of *A. butzleri* ED-1 (Accession ID AP012047). Further annotation and gene identification was done using Geneious 10.1 software (Bio-Matters). Annotation was done based on whole genome alignment with the ED-1 strain.

2.3. Gene targeting and CRISPR-mediated mutagenesis

To limit eliminate acetate or lactate metabolism in ED-1, we used KEGG (Kanehisa, 2000) to identify enzymes in these pathways that were annotated in the bacterium's genome. Alt-R Cas9 and Cpf1 (IDT) were used, and guide RNAs were designed according to the manufacturer's protocol and mapped to the novel strain genome (Table S1, Supplementary Information). For CAS9, 100 uM of Alt-R CRISPR-Cas9 crRNA and tracrRNA were mixed and heated to 95 °C for 5 min, allowed to cool back to room temperature (RT). The RNA mixture was then mixed with Alt-R Cas9 enzyme at a 1:1.2 molar ratio and allowed to form the RNP complex at RT for 20 min in a final volume of 20 ul. For Cpf1, 160 pmol crRNA was mixed directly with 126 pmol Cpf1 nuclease 2NLS and Phosphate Buffer Saline (pH 7.4) to a final volume of 5 uL and incubated at RT for 20 min to form the RNP complex ready for transformation. To transform the bacteria, 1 mL of log-phase bacteria (OD_{600} 0.3–0.6) was harvested and pelleted at 1000 g, resuspended to 80 uL with 10% ice cold glycerol and mixed with 2 uL of RNP complex in a 0.2 cm gap cuvette and pulsed once at 2500 V. 1 mL of Arcobacter Broth with antibiotics was immediately added post electroporation and bacteria were allowed to recover for 1 h at 30 °C under microaerophilic conditions. DNA was extracted from 500 uL of post-electroporation culture using Maxwell RSC Blood DNA kit (Promega), 400 uL was mixed with 200 uL of glycerol and placed in −80 °C as a stock and the remaining volume plated onto Arcobacter Broth agar plate with antibiotics to grow overnight at 30 °C and single colonies were picked for phenotypic validation the next day. DNA extracted from transformed cultures was amplified using Kapa HiFi PCR kit (Roche) subjected to T7 Endonuclease I digestion using Alt-R genome editing detection kit (IDT, [47]). 1.5% agarose gel stained with SYBR Green I (ThermoFisher) was prepared with 1× Tris-Acetate-EDTA and electrophoresis was performed at 50 mV for 60 min in 1× TAE running buffer (Mupid-exU).

2.4. Transcriptomic and phenotypic screening

Clonal transformed bacteria and untransformed control were grown on Arcobacter Broth media supplemented with pyruvate, antibiotics and 1 g/L acetate or lactate and incubated microaerobically at 30 °C for

12 h. RNA was extracted using Maxwell RSC RNA tissue kit (Promega) and cDNA strand synthesis was performed using SuperScript Reverse Transcriptase (Thermofisher) and random primers with the following protocol: 30 min at 16 °C, 42 °C for 1 h, 85 °C for 5 min in a thermal cycler. Samples for qPCR were prepared using a Quantitect kit (QIAGEN), as follows (volumes per individual sample, min. 3 samples prepared): 8.7 uL DEPC-water, 10 ul SYBR Green 2× Mastermix, 2 × 0.2 uL gene-specific primers (forward and reverse, 100 uM, see Supplementary Table 1), 1.3 uL cDNA (100 ng). RT-qPCR was performed on a StepOne Real-time PCR System (Applied Biosystems). For phenotypic assay, 100 uL of mid-log phase of clonal transformed bacteria were grown on basal media agar plate supplemented with antibiotics and 1 g/L acetate or lactate as sole carbon source and incubated microaerobically at 30 °C for 24 h. and checked for the colony growth.

2.5. MFC inoculation and operation

Two dual-chamber MFCs (Graphical abstract) were constructed by inserting stainless steel mesh (2 cm diameter) into each well of twelve-well plate (5 mL volume). Into six of wells acting as anodes, 2 mm thick carbon sponge (Toray carbon) discs were added to increase the surface area. Each disc was weighed while dry to calculate surface area (using a density of 4.51 g/cm^2) to obtain current density measurements (current/electrode surface area). Mixed bacterial cultures (from mangrove sediments) were grown at RT to OD_{600} 0.6 in the basal medium with 1 g/L acetate. All bacterial cultures were centrifuged at 5000 g for 5 min and resuspended in 1 mL basal medium. Six anodes of one well plate were filled with basal medium and either (1) mangrove sediment culture, (2) *A. butzleri* OK-1 or (3) no bacteria (negative control) in duplicates. Anodes of the second well plate were filled with *A. butzleri* OK-1 cultures and mutants grown on Arcobacter Broth with antibiotics and after 2 days replaced with basal medium containing either 500 mg/L acetate or lactate. Cathodes of all well plates were filled with 150 mM potassium ferricyanide (replaced whenever it became colourless). Each anode-cathode pair was connected with a 6 cm tube filled with 5 M KCl in 10% agar acting as a salt bridge, and stainless steel discs were connected with titanium wire and with 1000 Ohm resistors (Graphical abstract). Holes were made for media replacement and MFCs were stored in an anaerobic chamber (Ruskinn Bug Box, Baker) at 30 °C. Voltmeter was used to record potential between anode and cathode and current was calculated using Ohm's law. For the first well plate, acetate or lactate was added to six anodes at the concentrations of 0, 12.5, 25, 50, 100 and 200 ppm in 5 min increments and the current density was monitored. Experiment was repeated three times. After 4 weeks post inoculation of the second well plate, samples from two sponges were taken and subjected to specimen preparation using osmium protocol as described elsewhere [48].

3. Results

3.1. Isolation, sequencing and annotation

The genome of the novel strain *A. butzleri*, OK-1 (GenBank accession number CP041386), comprises a 2,196,900-bp circular chromosome of 27.2% GC content. The genome contains 2176 predicted protein-coding genes, 169 pseudogenes, 5 rRNA clusters and 55 tRNA genes (Fig. S1, see Table S5 for detailed information on annotation). After obtaining pure cultures of *A. butzleri* OK-1, we inoculated them in an MFC (Graphical abstract) to test their capability to attach to electrodes and visualised electrode samples under the SEM microscope (JEOL) (Fig. 1). Observations confirm that the new strain of *A. butzleri* can attach to electrodes, as indicated in (Fig. 1b). Similarly to ED-1 strain, no pili-encoding genes have been found; therefore, electron transfer might occur primarily via outer membrane cytochromes. These can be present in the outer membrane extensions, which can be seen in SEM images. Phenotypic screening indicate that *A. butzleri* OK-1 can use acetate and lactate as a carbon source, but cannot grow on succinate (Fig. S2).

3.2. Gene targeting for CRISPR-mediated mutagenesis

Using annotation, we identified 3 acetate kinase (*ackA*) and 1 lactate permease (*lctP*) genes (Fig. 2a) that were used for our targeted mutagenesis. Alignment of 3 acetate kinase genes (Fig. 2a), indicates only 72.9% sequence identity between all 3 genes, whereas *ackA*1 and *ackA*2 show 80.5% identity. However, when designing targets for Cas9, gRNA sequence and PAM 3′ was highly similar in three sequences, with 14–16 identical nucleotides (Fig. 2b). Therefore, we designed gRNA optimized for *ackA*2, but primers for subsequent qPCR and T7EI were designed so that all genes could be assayed. In the case of *lctP*, we decided to use Cpf1 (Fig. 2c).

3.3. Validation of CRISPR-mediated mutagenesis

T7EI digestion assay of the target regions demonstrates successful editing using Cpf1 (Fig. 3a). RT-qPCR also confirms the inhibition of target genes (Fig. 3b). Phenotypic screening of CRISPR-cpf1 mutants with impaired genes required for acetate metabolism (acetate kinase) could only grow when supplemented with lactate and not with acetate only, whereas the inverse was true for the lactate permease knockout, a gene involved in lactate metabolism (Fig. 3c).

3.4. MFC inoculation and operation

Observations of isolates indicate that *A. butzleri* OK-1 shows linear response to either acetate or lactate up to 50 mg/L (Fig. 4a), whereas mixed bacterial community showed high variability at all concentrations tested. Having mutants with desirable phenotypes, we then tested their ability to generate electricity. Upon inoculation with Arcobacter broth, we observed current generation in all strains, reaching 0.09 ± 0.02 A/m^2. Upon changing the media to basal media with acetate or lactate, current generation was only observed in the lactate or acetate mutants, respectively, with current densities reaching similar levels to the wild type cultures (Fig. 4b).

4. Discussion

Microbial Fuel Cells (MFCs) are an established technology allowing simultaneous metabolism of organic compounds and electricity production. Their performance depends on the activity of EAB that form biofilms on bioreactor anodes [13,49]. As electricity generators, today's MFCs achieve power densities of little more than 1000 mW/m^3 which limits their upscale applications [50]. However, as the current generated by a MFC is directly proportional to the energy content of the fuel, MFCs can be adapted as biosensors. The use of current-generating bacteria was first demonstrated by Karube and colleagues [51] where EAB at the anode of a microbial fuel cell oxidise the substrate as a fuel and the resulting electrons are directly transferred to the electrode to produce a detectable current, which correlates with BOD. Since then, amperometric biosensors have proven useful as alternative stable, online, real-time wastewater systems for monitoring general BOD, as well as toxic compounds, such as heavy metals [52–56]. While BOD detection is simply an indication of organic compounds and toxic compounds are mostly indicated by disturbance of the steady state signal output, a key limitation in this field has been single analyte detection [49].

In our study of limiting carbon metabolism in EAB, we were able to edit the genome of *A. butzleri* OK-1 using CRISPR techniques. Although Cpf1 system is considered more reliable for AT-rich genomes, [57], genome editing through Cas9 was also possible for *A. butzleri* OK-1, which has only 27.2% GC content. Our design condition was that the target genes should not be involved in electron donating/accepting pathways, e.g. dehydrogenase. Acetate kinase facilitates the production acetyl-CoA by phosphorylating acetate. Lactate permease transports

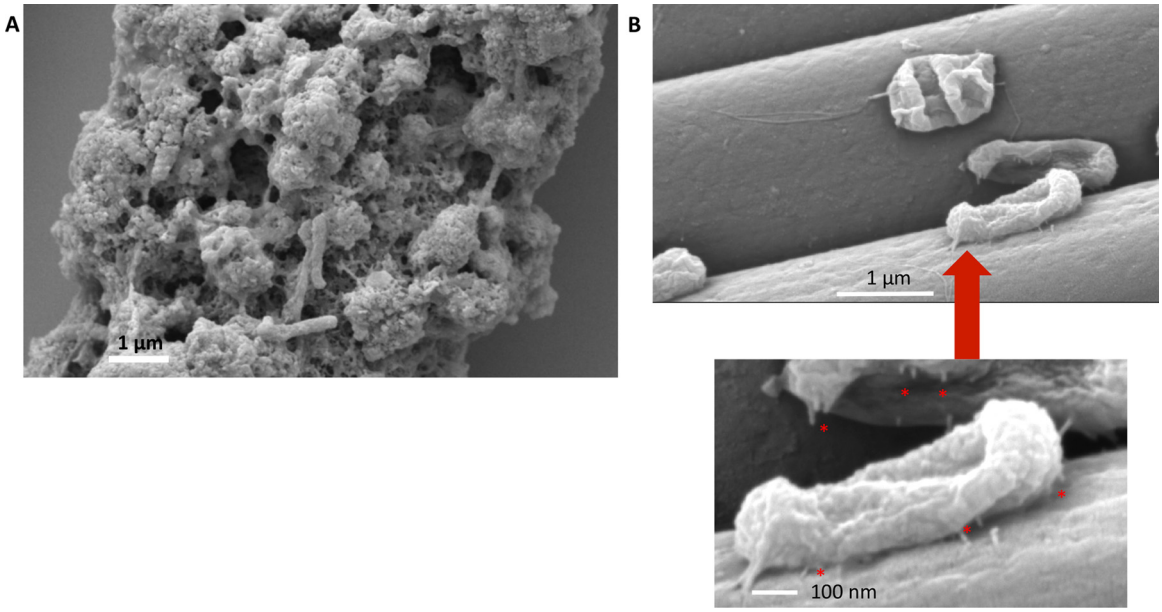

Fig. 1. SEM images of *A. butzleri* OK-1 **A**, Biofilm overview; **B**, individual cells attached to carbon sponge electrode with outer membrane extensions marked by asterisks and seen in magnification.

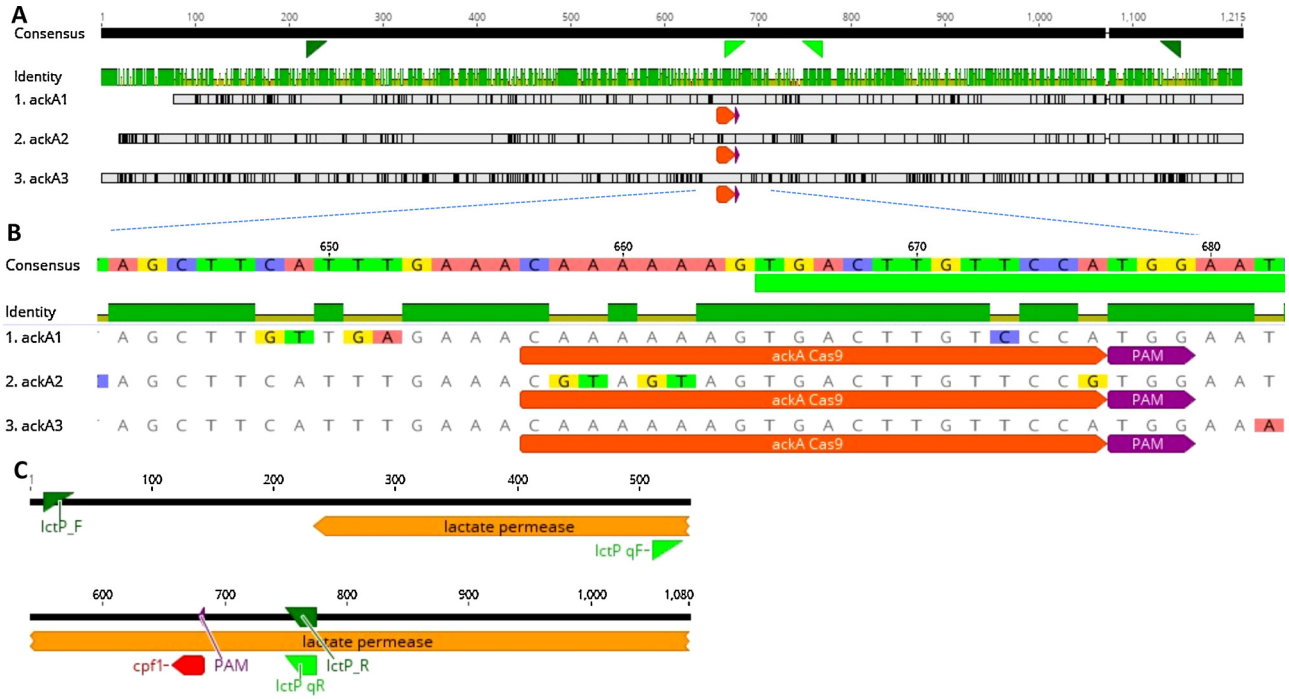

Fig. 2. **A**, Alignment of *ackA* genes identified in *A. butzleri* OK-1 orange arrow indicates Cas9 guide sequence, purple arrows indicate PAM sequence, dark green arrows indicate primers used for T7EI assay, whereas light green arrows indicate primers used for qPCR; **B**, Alignment of Cas9 guide and PAM sequences in *ackA* genes; **C** Alignment of *lctP* gene with Cpf1 guide, PAM and primers.

lactate into the cell via proton pump. Resulting mutants were generating current at the same level as the wild type strain, indicating electrogenic properties of *A. butzleri* remained unaffected by genome editing (Fig. 3b). This provides a foundation for further engineering, with the possibility of introducing new metabolic genes in diverse EAB that might produce stronger current or detect different analytes. Such biosensors could be applied in many industries, in addition to wastewater monitoring, such as the food, beverage, or pharmaceutical industries for online, real-time quality control.

Moreover, there is a growing interest in utilizing electrogenic bacteria in bioprocesses. As EAB cannot only generate, but also receive electrons from electrode, applying electricity in fermentative reactors shows 3–4 improvement of desired bioproduction, as well as reduction of unwanted byproducts [58–60], making electrofermentation (EF) attractive for industrial use. EF may also be used to directly control and switch the chassis' metabolism. It has been recently demonstrated that the redox switch can be inserted into non-EAB, e.g. *E. coli* genome to control its gene expression in response to changes in electrode potential [61]. Having a tightly controlled gene regulation system – electrogenetics – would allow introducing multilevel bioprocessing, enabling to perform antagonistic reactions serially in the same reactor, just by means of potential regulation [62]. Future work could also result in

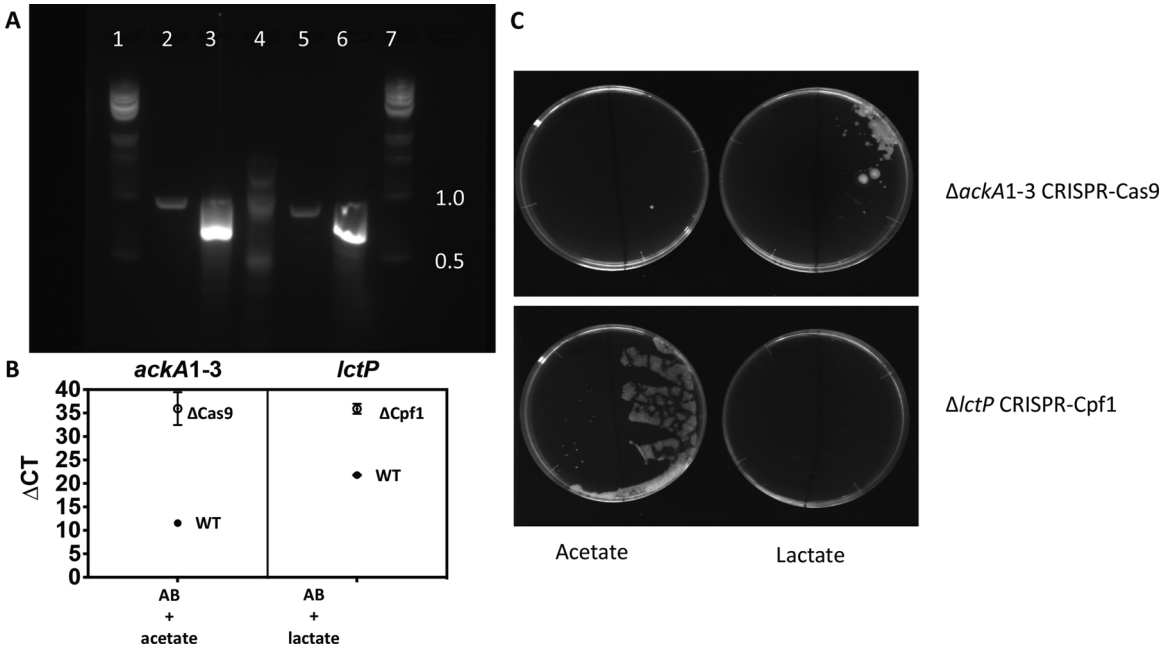

Fig. 3. **A**, T7EI assay of CRISPR - edited *A. butzleri* OK-1. Lanes are as follows: **1,7** 1kb ladder; **2** *ackA* control; **3** *ackA* mutant; **4** 100 bp ladder; **5** *lctP* control; **6** *lctP* mutant. **B**, RT-qPCR sequencing of CRISPR-edited *A. butzleri* grown in rich medium with with acetate and lactate. **WT**, wild-type. Error bars indicate SD ($n = 3$, in the case of *ackA* WT and *lctP* WT, SD values are 0.2 and 0.14, respectively). **C**, Phenotypic screening of obtained mutants. In the case of *ackA*, single colony was observed on acetate agar, but showed different morphology and PCR with *A. butzleri*-specific 16S primers did not yield any product.

pathway insertions into the *A. butzleri* OK-1 genome to develop desirable metabolism only where a specific carbon source is present in the media. Moreover, it may be possible to engineer the OK-1 strain to grow on unusual carbon sources, or to break down recalcitrant substrates, such as lignocellulose, without the subsequent loss of desirable degradation products (saccharides) that could be used in downstream processing.

5. Conclusions

We were completely successful in producing mutations at the target CRISPR cleavage sites for either *ackA* or *lctP*, the former being present in 3 copies, with some sequence divergence (Figs. 2 and 3). The successful knockout of acetate metabolism could be attributed to the off-target activity. Although CRISPR-Cas9 has already been used to edit another EAB genome [37], no test on current generation in mutants have been conducted. This is also the first time EAB, as well as a gram-negative proteobacterium, has been modified with CRISPR-Cpf1. As some *Arcobacter* spp. have been associated with virulence, whilst other work shows suppressant effects of MFCs on pathogenic organisms [63,64], it may prove useful to study these relationships and utilize electrogenicity to combat pathogen outbreaks. Given its complete genome annotation, *A. butzleri* OK-1 can serve as a platform to study genome-editing effects on extracellular electron transfer. Furthermore, miniaturization of MFCs based on well plate array, together with microfluidics would allow multiple compounds to be screened simultaneously using metabolic mutants of various EAB growing in MFC. Similar work was already done with single EAB, where logic AND gate was created by allowing electricity generation only upon the presence of two input signals [65]. This could lead to the development of self-controlled factories, where various compound detection and signal transduction could trigger appropriate response.

Authors' contributions

Lukasz Szydlowski: Conceptualization, Methodology, Original draft preparation, Writing – Reviewing and Editing

Tammy Chih Ting Lan: Data curation, Visualization, Investigation.

Noriko Shibata: Visualization.

Igor Goryanin: Supervision.

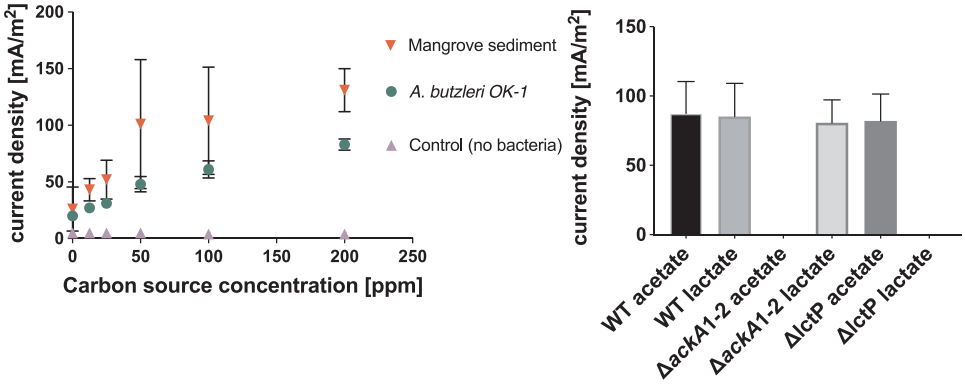

Fig. 4. Current densities of MFC well plates inoculated with **A**, *A. butzleri* OK-1 isolate, and mangrove sediment culture, **B**, *A. butzleri* OK-1, *ackA*1-2 and *lctP* mutants. Error bars indicate SD ($n = 6$).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.enzmictec.2020.109564.

References

[1] Y. Moreno, S. Botella, J.L. Alonso, M.A. Ferrús, M. Hernández, J. Hernández, Specific detection of arcobacter and campylobacter strains in water and sewage by PCR and fluorescent in situ hybridization, Appl. Environ. Microbiol. 69 (2003) 1181–1186.

[2] J. Snaidr, R. Amann, I. Huber, W. Ludwig, K.H. Schleifer, Phylogenetic analysis and in situ identification of bacteria in activated sludge, Appl. Environ. Microbiol. 63 (1997) 2884–2896.

[3] T.L. Maugeri, C. Gugliandolo, M. Carbone, D. Caccamo, M.T. Fera, Isolation of Arcobacter spp. from a brackish environment, New Microbiol. 23 (2000) 143–149.

[4] E.W. Rice, M.R. Rodgers, I.V. Wesley, C.H. Johnson, S.A. Tanner, Isolation of arcobacter butzleri from ground water, Lett. Appl. Microbiol. 28 (1999) 31–35.

[5] M.T. Fera, T.L. Maugeri, C. Gugliandolo, C. Beninati, M. Giannone, E. La Camera, M. Carbone, Detection of Arcobacter spp. in the coastal environment of the Mediterranean Sea, Appl. Environ. Microbiol. 70 (2004) 1271–1276.

[6] A.L. Diéguez, A. Pérez-Cataluña, N. Salas-Massó, J.L. Romalde, A. Lema, S. Balboa, M.J. Figueras, Revisiting the taxonomy of the genus *Arcobacter: getting order from the chaos*, Front. Microbiol. 9 (2018).

[7] B. Thamdrup, R. Rosselló-Mora, R. Amann, Microbial manganese and sulfate reduction in black sea shelf sediments, Appl. Environ. Microbiol. 66 (2000) 2888–2897.

[8] I.S. Chang, H. Moon, O. Bretschger, J.K. Jang, H.I. Park, K.H. Nealson, B.H. Kim, Electrochemically active bacteria (EAB) and mediator-less microbial fuel cells, J. Microbiol. Biotechnol. 16 (2006) 163–177.

[9] S. Sharma, G. Cavallaro, A. Rosato, A systematic investigation of multiheme c-type cytochromes in prokaryotes, J. Biol. Inorganic Chem. 15 (2010).

[10] G. Reguera, K.D. McCarthy, T. Mehta, J.S. Nicoll, M.T. Tuominen, D.R. Lovley, Extracellular electron transfer via microbial nanowires, Nature 435 (2005) 1098–1101.

[11] K. Rabaey, Universidade estadual paulista faculdade de ciências e letras campus araraquara, Monografia 70 (2004) 5373–5382.

[12] C. Santoro, C. Arbizzani, B. Erable, I. Ieropoulos, Microbial fuel cells: from fundamentals to applications, Rev. J. Power Sources 356 (2017) 225–244.

[13] R.M. Allen, H.P. Bennetto, Microbial fuel cells: electricity production from carbohydrates, Appl. Biochem. Biotechnol. 39 (1993) 27–40.

[14] B.E. Logan, B. Hamelers, R. Rozendal, U. Schroder, J. Keller, S. Freguia, P. Aelterman, W. Verstraete, K. Rabaey, Microb. Fuel Cells: Methodol. Technol. 40 (2006) 5181–5192.

[15] V. Fedorovich, M.C. Knighton, E. Pagaling, F.B. Ward, A. Free, I. Goryanin, Novel electrochemically active bacterium phylogenetically related to Arcobacter butzleri, isolated from a microbial fuel cell, Appl. Environ. Microbiol. 75 (2009) 7326–7334.

[16] L. Kiseleva, S.K. Garushyants, H. Ma, D.J.W. Simpson, V. Fedorovich, M.F. Cohen, I. Goryanin, Taxonomic and functional metagenomic analysis of anodic communities in two pilot-scale microbial fuel cells treating different industrial wastewaters, J. Integr. Bioinform. 12 (2015) 273.

[17] I.V. Khilyas, A.A. Sorokin, L. Kiseleva, D.J. Simpson, V. Fedorovich, M.R. Sharipova, M. Kainuma, M.F. Cohen, I. Goryanin, Comparative metagenomic analysis of electrogenic microbial communities in differentially inoculated swine wastewater-fed microbial fuel cells, Scientifica 2017 (2017).

[18] L. Szydlowski, A. Sorokin, O. Vasieva, S. Boerner, V. Fedorovich, I. Goryanin, Evolutionary dynamics of microbial communities in bioelectrochemical systems, BioRxiv (2019).

[19] F. Rovetto, A. Carlier, A.M. Van Den Abeele, K. Illeghems, F.V. Nieuwerburgh, L. Cocolin, K. Houf, Characterization of the emerging zoonotic pathogen Arcobacter thereius by whole genome sequencing and comparative genomics, PLOS ONE 12 (2017) 1–27.

[20] A.M. Van den Abeele, D. Vogelaers, J. Van Hende, K. Houf, Prevalence of *Arcobacter species among humans, Belgium, 2008–2013*, Emerg. Infect. Dis. 20 (2014) 1731–1734.

[21] L. Collado, M.J. Figueras, Taxonomy epidemiology, and clinical relevance of the genus Arcobacter, Clin. Microbiol. Rev. 24 (2011) 174–192.

[22] E. Arguello, C.C. Otto, P. Mead, N.E. Babady, Bacteremia caused by Arcobacter butzleri in an immunocompromised host, J. Clin. Microbiol. 53 (2015) 1448–1451.

[23] H. Toh, V.K. Sharma, K. Oshima, S. Kondo, M. Hattori, F.B. Ward, A. Free, T.D. Taylor, Complete genome sequences of Arcobacter butzleri ED-1 and Arcobacter sp. Strain L both isolated from a microbial fuel cell, J. Bacteriol. 193 (2011) 6411–6412.

[24] I. Martinez-Malaxetxebarria, R. Muts, L. van Dijk, C.T. Parker, W.G. Miller, S. Huynh, W. Gaastra, J.P.M. van Putten, A. Fernandez-Astorga, M.M.S.M. Wösten, Regulation of energy metabolism by the Extracytoplasmic Function (ECF) (Factors of Arcobacter butzleri, PLoS ONE 7 (2012) e44796.

[25] Y. Ishino, H. Shinagawa, K. Makino, M. Amemura, A. Nakatura, Nucleotide sequence of the iap gene responsible for alkaline phosphatase isoenzyme conversion in Escherichia coli, and identification of the gene product, J. Bacteriol. 169 (1987) 5429–5433.

[26] J. Selengut, E.F. Mongodin, K.E. Nelson, A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/cas subtypes exist in prokaryotic genomes, PLoS Comput. Biol. 1 (2005) 0474–0483.

[27] F.A. Ran, P.D. Hsu, J. Wright, V. Agarwala, D.A. Scott, F. Zhang, Genome engineering using the CRISPR-Cas9 system, Nat. Protoc. 8 (2013) 2281–2308.

[28] J.A. Doudna, E. Charpentier, The new frontier of genome engineering with CRISPR-Cas9, Science 346 (2014).

[29] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, P.D. Hsu, X. Wu, W. Jiang, L.a. Marraffini, Multiplex genome engineering using CRISPR/cas systems, Science 339 (2013) 819–823.

[30] B. Zetsche, J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, S.E. Volz, J. Joung, J. van der Oost, A. Regev, E.V. Koonin, F. Zhang, M. Ian, J.V.D. Oost, A. Regev, E.V. Koonin, F. Zhang, cpf1 is a single rna-guided endonuclease of a class 2 crispr-cas system, Cell (2015) 759–771.

[31] R. Yao, D. Liu, X. Jia, Y. Zheng, W. Liu, Y. Xiao, CRISPR-Cas9/Cas12a biotechnology and application in bacteria, Synth. Syst. Biotechnol. 3 (2018) 135–149.

[32] Y. Jiang, B. Chen, C. Duan, B. Sun, J. Yang, S. Yang, Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system, Appl. Environ. Microbiol. 81 (2015) 2506–2514.

[33] J. Liu, Y. Wang, Y. Lu, P. Zheng, J. Sun, Y. Ma, Development of a CRISPR/Cas9 genome editing toolbox for Corynebacterium glutamicum, Microb. Cell Fact. 16 (2017) 1–17.

[34] K.N. McAllister, L. Bouillaut, J.N. Kahn, W.T. Self, J.A. Sorg, Using CRISPR-Cas9-mediated genome editing to generate C. difficile mutants defective in selenoproteins synthesis, Sci. Rep. 7 (2017) 1–12.

[35] X. Song, H. Huang, Z. Xiong, L. Ai, S. Yang, CRISPR-Cas9^{D10A} nickase-assisted genome editing in *Lactobacillus casei*. Appl. Environ. Microbiol. 83 (2017) 1–13.

[36] J. Behler, D. Vijay, W.R. Hess, M.K. Akhtar, CRISPR-based technologies for metabolic engineering in cyanobacteria, Trends Biotechnol. 36 (2018) 996–1010.

[37] Y. Suzuki, A. Kouzuma, K. Watanabe, CRISPR/Cas9-mediated genome editing of *Shewanella oneidensis MR-1 using a broad host-range pBBR1-based plasmid*, J. Gen. Appl. Microbiol. (2019).

[38] Y. Jiang, F. Qian, J. Yang, Y. Liu, F. Dong, C. Xu, B. Sun, B. Chen, X. Xu, Y. Li, R. Wang, S. Yang, CRISPR-Cpf1 assisted genome editing of Corynebacterium glutamicum, Nat. Commun. 8 (2017) 1–11.

[39] S. Kim, D. Kim, S. Cho, J. Kim, J.-S. Kim, Highly efficient RNA-guide genome editing, Genome Res. 128 (2014) 1–32.

[40] X. Liang, J. Potter, S. Kumar, Y. Zou, R. Quintanilla, M. Sridharan, J. Carte, W. Chen, N. Roark, S. Ranganathan, N. Ravinder, J.D. Chesnut, Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection, J. Biotechnol. 208 (2015) 44–53.

[41] S. Lin, B.T. Staahl, R.K. Alla, J.A. Doudna, Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery, eLife 3 (2014) e04766.

[42] K. Schumann, S. Lin, E. Boyer, D.R. Simeonov, M. Subramaniam, R.E. Gate, G.E. Haliburton, C.J. Ye, J.A. Bluestone, J.A. Doudna, A. Marson, Generation of knock-in primary human T cells using Cas9 ribonucleoproteins, Proc. Natl. Acad. Sci. USA 112 (2015) 10437–10442.

[43] T. Gaj, B.T. Staahl, G.M. Rodrigues, P. Limsirichai, F.K. Ekman, J.A. Doudna, D.V. Schaffer, Targeted gene knock-in by homology-directed genome editing using Cas9 ribonucleoprotein and AAV donor delivery, Nucl. Acids Res. 45 (2017) 1–11.

[44] B.A. Lone, S.K.L. Karna, F. Ahmad, N. Shahi, Y.R. Pokharel, CRISPR/Cas9 System: a bacterial tailor for genomic engineering, Genet. Res. Int. 2018 (2018).

[45] N.V. Narendranath, K.C. Thomas, W.M. Ingledew, Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium, J. Ind. Microbiol. Biotechnol. 26 (2001) 171–177.

[46] F. Raposo, R. Borja, J.A. Cacho, J. Mumme, K. Orupõld, S. Esteves, J. Noguerol-Arias, S. Picard, A. Nielfa, P. Scherer, I. Wierinck, E. Aymerich, C. Cavinato, D.C. Rodriguez, N. García-Mancha, P.N.T. Lens, V. Fernández-Cegrí, First international comparative study of volatile fatty acids in aqueous samples by chromatographic techniques: evaluating sources of error, TrAC – Trends Anal. Chem. 51 (2013) 127–143.

[47] L. Vouillot, A. Th’elie, N. Pollet, Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases, G3: Genes Genomes Genet. 5 (2015) 407–415.

[48] E.R. Fischer, B.T. Hansen, V. Nair, F.H. Hoyt, D.W. Dorward, Scanning electron microscopy, Curr. Protoc. Microbiol. (2012) 1–16.

[49] X.C. Abrevaya, N.J. Sacco, M.C. Bonetto, A. Hilding-Ohlsson, E. Cortón, Analytical applications of microbial fuel cells. Part II: Toxicity, microbial activity and quantification, single analyte detection and other uses, Biosens. Bioelectron. 63 (2015) 591–601.

[50] Y. Fan, H. Hu, H. Liu, Enhanced coulombic efficiency and power density of air-cathode microbial fuel cells with an improved cell configuration, J. Power Sources 171 (2007) 348–354.

[51] I. Karube, T. Matsunaga, S. Mitsuda, S. Suzuki, Microbial electrode bod sensors, Biotechnol. Bioeng. 19 (1977) 1535–1547.

[52] M. Kim, S.M. Youn, S.H. Shin, J.G. Jang, S.H. Han, M.S. Hyun, G.M. Gadd, H.J. Kim, Practical field application of a novel BOD monitoring system, J. Environ. Monit. 5 (2003) 640–643.

[53] M. Di Lorenzo, T.P. Curtis, I.M. Head, K. Scott, A single-chamber microbial fuel cell as a biosensor for wastewaters, Water Res. 43 (2009) 3145–3154.

[54] G.-X. Yang, Y.-M. Sun, X.-Y. Kong, F. Zhen, Y. Li, L.-H. Li, T.-Z. Lei, Z.-H. Yuan, G.-Y. Chen, Factors affecting the performance of a single-chamber microbial fuel cell-type biological oxygen demand sensor, Water Sci. Technol. 68 (2013) 1914–1919.

[55] A. Kaur, J.R. Kim, I. Michie, R.M. Dinsdale, A.J. Guwy, G.C. Premier, Microbial fuel cell type biosensor for specific volatile fatty acids using acclimated bacterial communities, Biosens. Bioelectron. 47 (2013) 50–55.

[56] M. Di Lorenzo, A.R. Thomson, K. Schneider, P.J. Cameron, I. Ieropoulos, A small-scale air-cathode microbial fuel cell for on-line monitoring of water quality,

Biosens. Bioelectron. 62 (2014) 182–188.
[57] D.E. Watkins-Chow, G.K. Varshney, L.J. Garrett, Z. Chen, E.A. Jimenez, C. Rivas, K.S. Bishop, R. Sood, U.L. Harper, W.J. Pavan, S.M. Burgess, Highly efficient Cpf1-mediated gene targeting in mice following high concentration pronuclear injection, G3: Genes Genomes Genet. 7 (2017) 719–722.
[58] O. Choi, T. Kim, H.M. Woo, Y. Um, Electricity-driven metabolic shift through direct electron uptake by electroactive heterotroph Clostridium pasteurianum, Sci. Rep. 4 (2014).
[59] R. Moscoviz, J. Toledo-Alarcón, E. Trably, N. Bernet, Electro-fermentation: how to drive fermentation using electrochemical systems, Trends Biotechnol. 34 (2016) 856–865.
[60] S. Roy, A. Schievano, D. Pant, Electro-stimulated microbial factory for value added product synthesis, Bioresour. Technol. 213 (2016) 129–139.
[61] T. Tschirhart, E. Kim, R. Mckay, H. Ueda, H.C. Wu, A.E. Pottash, A. Zargar, A. Negrete, J. Shiloach, G.F. Payne, W.E. Bentley, Electronic control of gene expression and cell behaviour in Escherichia coli through redox signalling, Nat. Commun. 8 (2017) 1–11.
[62] A. Hirose, A. Kouzuma, K. Watanabe, Towards development of electrogenetics using electrochemically active bacteria, Biotechnol. Adv. (2019) 0–1.
[63] I. Ieropoulos, G. Pasternak, J. Greenman, Urine disinfection and in situ pathogen killing using a Microbial Fuel Cell cascade system, PLoS ONE 12 (2017) 1–12.
[64] O. Vasieva, A. Sorokin, L. Szydlowski, I. Goryanin, Do microbial fuel cells have antipathogenic properties? J. Comput. Sci. Syst. Biol. 12 (2019) 57–70.
[65] Y. Hu, Y. Yang, E. Katz, H. Song, Programming the quorum sensing-based AND gate in *Shewanella oneidensis for logic gated-microbial fuel cells*, Chem. Commun. 51 (2015) 4184–4187.