Taxonomic and functional metagenomic analysis of anodic communities in two pilot-scale microbial fuel cells treating different industrial wastewaters

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Summary

The combined processes of microbial biodegradation accompanied by extracellular electron transfer make microbial fuel cells (MFCs) a promising new technology for cost-effective and sustainable wastewater treatment. Although a number of microbial species that build biofilms on the anode surfaces of operating MFCs have been identified, studies on the metagenomics of entire electrogenic communities are limited. Here we present the results of whole-genome metagenomic analysis of electrochemically active robust anodic microbial communities, and their anaerobic digester (AD) sludge inocula, from two pilot-scale MFC bioreactors fed with different distillery wastewaters operated under ambient conditions in distinct climatic zones. Taxonomic analysis showed that *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were abundant in AD sludge from distinct climatic zones, and constituted the dominant core of the MFC microbiomes. Functional analysis revealed species involved in degradation of organic compounds commonly present in food industry wastewaters. Also, accumulation of methanogenic *Archaea* was observed in the electrogenic biofilms, suggesting a possibility for simultaneous electricity and biogas recovery from one integrated wastewater treatment system. Finally, we found a range of species within the anode communities possessing the capacity for extracellular electron transfer, both via direct contact and electron shuttles, and show differential distribution of bacterial groups on the carbon cloth and activated carbon granules of the anode surface. Overall, this study provides insights into structural shifts that occur in the transition from an AD sludge to an MFC microbial community and the metabolic potential of electrochemically active microbial populations with wastewater-treating MFCs.

1 Introduction

Amongst proposed green energy technologies, microbial fuel cells (MFCs) hold promise as an efficient and cost-effective solution for global wastewater treatment. Within an MFC,

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anaerobically respiring microorganisms degrade organic compounds and donate electrons to an external circuit, thereby coupling removal of organics with electrical power production [1]. These systems have proved efficient in laboratory-scale settings, and research into scaled-up designs for treatment of industrial and municipal wastewaters is ongoing [2, 3, 4]. Once integrated into waste treatment processes, MFC technology is expected to have an advantage over aerobic wastewater treatment systems due to low biomass production and reduced energy consumption. Also, unlike most biogas-producing anaerobic digesters, MFC bioreactors are able to operate in ambient temperatures and do not require the treatment of the end products generated in the cell [1, 5].

Thus far, MFC scaling up and development has mostly focused on optimizing reactor design and identifying inexpensive efficient materials, with the aim of reducing cost and improving efficiency to levels required for large-scale wastewater treatment [3, 4, 6, 7, 8, 9]. However, better knowledge of microbial components will be necessary to achieve optimal performance [9]. Maximizing the range and rate of complex substrate subject to degradation is a critical requirement and is highly determined by metabolic capabilities of inhabiting microorganisms. Therefore, it is essential to identify active populations active within mixed microbial communities with the intent of designing microbial inoculums for scaled-up MFCs. The most efficient community would be expected to contain a significant pool of genes involved in biodegradation and extracellular electron transfer.

Bacteria within MFCs can transfer electrons to anodic materials either through direct contact or via exuded soluble electron shuttling compounds [10, 11]. External electron transfer mechanisms are particularly well-characterized for the genera *Geobacter* and *Shewanella*. Electron transfer capabilities can vary substantial even between closely related *Geobacter* species [12]. Fine-scale transcriptional analysis of anode biofilms of *Geobacter sulfurreducens* found differences in gene expression across the biofilm depths [13] whereas mixed and pure culture biofilms of *Geobacter anodireducens* were found to be active only on the outer layer of the biofilm, with the dead layer in contact with the anode surface serving as an electrically conductive matrix [14]. Metatranscriptomic analysis of mixed community anode biofilms demonstrated significant changes in gene expression by associated sulfate-reducing *Desulfobulbaceae* in response to changes in electron transfer activity [15].

Previous metagenomic analysis of MFCs has focused on low volume capacity (<0.5L) laboratory-scale MFCs [16, 17]. Here we report whole-genome metagenomic analysis of anodic communities from two large 60-64 L capacity distillery wastewater-fed MFCs designed for use in practical-scale treatment as part of a modular MFC system. Examination of the bio-electroremediation potential of the communities and their source inoculums shows that although the MFC bioreactors differed both in design, inoculum and feed composition they developed a common core community. Both MFC communities revealed shifts in population structure from their respective inoculums that occurred during operation of the bioreactors under real-world conditions.

2 Methods

2.1 MFC operation and sampling

Two pilot-scale MFC bioreactors were produced by MPower World Ltd (Scotland, UK), and tested for electricity generation and COD removal with real wastewater under ambient conditions. The construction of the MFCs was based on a horizontal flow bioreactor [18]. The first reactor (UK), located at Elvingston Science Park, Scotland, had a 64 L active volume capacity and was fed whisky pot ale from a local distillery. The second reactor (JP) with 60 L active volume capacity was operated at OIST Graduate University (Japan) (Figure 1), and fed

with wastewater from an awamori distillery (Mizuho, Naha). Feeds were diluted to achieve an average COD of 10 g L^{-1}. Both reactors had four pairs of electrodes, air-breathing cathodes and were inoculated with sludge obtained from anaerobic digesters at the local wastewater treatment plants. The UK reactor operated continuously in closed circuit while the JP reactor was maintained on a 12 h closed, 12 h open circuit cycle. COD removal efficiency and power generation were measured to assess the MFC performance. pH of the UK bioreactor was adjusted by using recycled effluent for dilution. This increased the distillery influent pH from 4.0 to 5.5 as verified by a pH meter (Thermo-scientific Orion^{TM} 5-Star Plus, USA) at the inflow sample port. The pH of the JP bioreactor was adjusted to 6.2 using $NaHCO_3$ powder dissolved in influent solution and verified with a pH meter (Horiba D-51, Japan).

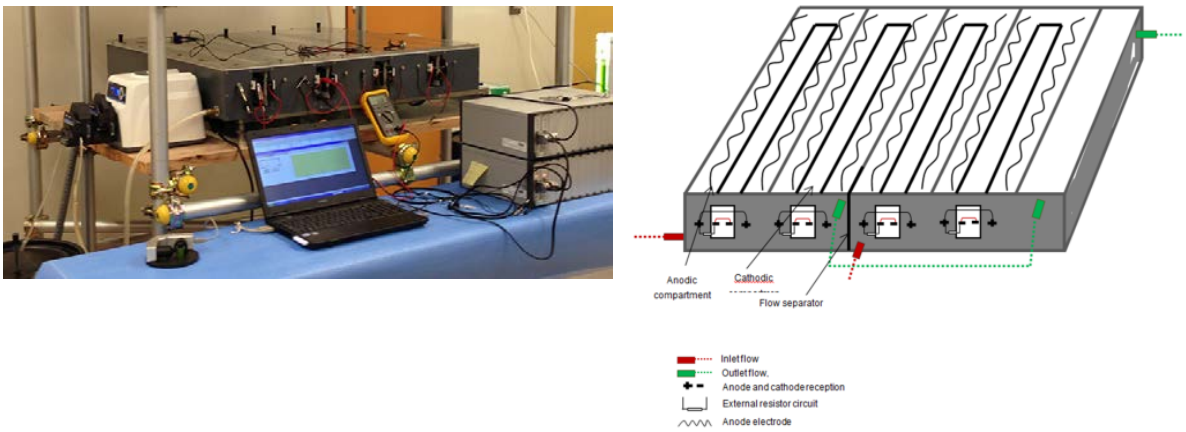

Figure 1: The awamori wastewater-fed microbial fuel cell bioreactor and associated equipment, Okinawa, Japan (left) and schematic drawing of the MFC showing fluid flow and electrical components (right).

2.2 Sample collection, DNA extraction and sequencing

The UK bioreactor was inoculated with AD granular sludge from a whisky wastewater-treatment plant (Elgin, UK), and had been operated for 90 d at the time of sample collection. The second bioreactor (JP) was inoculated with AD granular sludge from a wastewater treatment facility located within Orion brewery (Okinawa, Japan) and operated 70 d before sampling. The anode biofilms for metagenomic analysis were collected in different manners depending on location due to dissimilar MFC configurations. The layer of biofilm was scraped off the anodic surface of the UK bioreactor; whereas a piece of carbon felt with the attached granules was cut off the anode of the JP bioreactor. Also, 10 mL of the corresponding inoculum sludge was collected as a reference sample for each MFC. All samples were immediately preserved in LifeGuard Soil Preservation Solution, following the manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA, USA). Total genomic DNA was extracted from each MFC reference initial inoculum sludge, the anodic biofilm of the UK MFC, and separately from carbon felt and carbon granules of the JP MFC using PowerMax soil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). DNA quality was assessed by spectrophotometric analysis and agarose-gel electrophoresis. Approximately 5 µg of quality-checked DNA was used for library construction and sequencing with a Roche 454 GS FLX Titanium (454 Life Science, Branford, CT, U SA) according to the manufacturer's protocols [19]. Low quality bases and primers were end-trimmed. Reads were assembled *de novo* by GS DeNovo Assembler version 2.8. All contigs longer than 500 bp were selected for further analysis.

2.3 Metagenomic DNA sequence analysis

The resultant contigs and singletons were uploaded to MG-RAST (MetaGenome Rapid Annotation with Subsystem Technology) [20]: Japan MFC AD sludge (4540100.3, 4540109.3); Japan MFC anode samples (4539687.3, 4540099.3); Japan MFC anode cloth and granules (4540129.3, 4540130.3, 4540190.3, 4540191.3); UK MFC AD sludge (4540110.3, 4543057.3); UK MFC anode sample (4539688.3, 4540098.3), where additional removal of artificial replicates and filtering for *H. sapiens* sequences was performed. Unassembled JP carbon cloth and carbon granule samples were also uploaded to MG-RAST. While MG-RAST analysis is suited well for reads of the same length, it does not provide a way to calculate the phylogenetic distribution in metagenomic samples having mixed sequences of different lengths and coverage. Therefore, after MG-RAST BLAT search, all resulting files were downloaded from the server and analyzed independently.

2.3.1 Phylogenetic annotation

Phylogenetic annotation of each metagenome was performed based on 16S rRNA gene fragments and sequence reads. Ribosomal RNA sequences for each metagenome after clustering (97% identity) were downloaded from MG-RAST server and classified by SILVA Aligner (with the minimum identity with query sequence 0.85), and RDP classifier (with the minimum confidence score of 0.8) [21]. SILVA Aligner was utilized as the primary source of classification, while RDP classification was assigned to the sequence only when not classifiable by SILVA.

To perform phylogenetic annotation on all metagenomic sequences and calculate the phylogenetic ranks abundances, results of BLAT search against RefSeq database was utilized. To assign taxonomy to the individual sequences the lowest common ancestor (LCA) approach was used. Nearest neighbors of the particular sequence were determined as hits with alignment length of at least 50 bp, and with maximum permitted difference of 10% from the maximum identity, but with identity not less than 60%, and BLAT E-value of at least 10^{-5}. These selected hits were used for LCA, and the common part of taxonomy for nearest neighbors was assigned to the metagenomic sequence. Abundance of each phylogenetic rank was calculated as the number of shotgun reads assigned to the particular phylogenetic rank, where abundance for a contig is the number of reads included in this contig. All taxonomic ranges were assigned according to NCBI Taxonomy [22, 23].

2.3.2 Functional annotation

All found proteins were assigned to both Clusters of Orthologous Genes (COG) [24] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database [25] by MG-RAST. Results of BLAT search against COG and KEGG databases were downloaded from MG-RAST API. COG or KO IDs were assigned to the particular protein, if it had sequence identity more than 60% and alignment length of at least 80 bp.

Abundance for individual COG or KO was calculated as the number of reads, where the particular COG is present, multiplied by the average coverage of the sequence. The abundance of each category or level is the sum of abundances for each COG or KO in the group. To study the most abundant functions on anodic biofilms we chose all the COGs in JP and UK anode metagenomic samples with abundance of more than 0.25%, and compared their abundances with the ones in the JP and UK inoculums.

2.3.3 Functions important for MFC productivity

Proteins involved in phenazine-1-carboxylate biosynthesis pathway were extracted from MetaCyc database [26] while proteins involved in transduction of electrons to anode were extracted from the total 35 found in the literature [27, 28, 29, 30, 31, 32]. Protein sequences mentioned above were used for BLAST homology searches against both anode metagenome sample protein databases with 45% sequence similarity, alignment length of 60 aa, and expectation of 10^{-5}.

Genes involved in acetate utilization were extracted from KEGG, COG and CDD: K00625 – phosphate acetyltransferase; K01895 – acetyl-CoA synthetase; K00925 – acetate kinase; K14393, and PRK09395 (CDD ID) – acetate permease *actP*; K01637 and COG2224 – isocitrate lyase; K01638 – malate synthase. In the case of CDD, alignment matches were revealed by HMMer search against metagenome protein databases with a bitscore cutoff of 120. Taxonomy for each found hit was determined as phylogenetic rank of the sequence, where hits were found. Found genes were plotted on NCBI taxonomy tree and visualized with iTOL [23].

3 Results and Discussion

3.1.1 MFC operation and microbial sample collection

Two multi-electrode pilot MFC bioreactors of similar design [18] successfully treated wastewater from local distilleries and generated electrical power; one in Edinburgh, Scotland (UK), the other in Okinawa, Japan (JP). Each MFC was independently inoculated with a local AD sludge and maintained under ambient conditions with average temperatures of 10°C and 27°C for the UK and JP reactor, respectively. The UK bioreactor was fed whisky pot ale wastewater while the JP bioreactor was fed rice wash and spentwash mixed awamori distillery wastewater. The pH level in UK bioreactor was maintained at 5.5 in order to reduce possible methanogenic activities that could potentially decrease electrical power output, while it was adjusted to pH 6.2 in the JP MFC in order to increase digestion of organics.

Table 1: Microbial fuel cell bioreactor characteristics.

	UK MFC	**JP MFC**
Working anode chamber volume (L)	64	60
System flow (m^3 d^{-1})	0.01	0.05
Max COD inlet concentration (g L^{-1})	15	10
Max COD outlet concentration (g L^{-1})	4.2	2
Max COD removal efficiency (%)	72	80
Avg. hydraulic retention time (d)	6.3	4
Shortest HRT (d)	5	3.4
Max cell voltage (mV)	500	650
Max cell current, open circuit (mA)	85	120
Maximum direct electricity (W m^{-3})	2.56	10.36
Cathode power by surface (W m^{-2})	0.0133	0.0164
Cathode surface area (m^2)	19.2	37.9
Evaluation period (d)	90	70

UK MFC, MFC operating at Edinburgh University, UK; JP MFC, MFC operating at OIST Graduate University, Okinawa, Japan.

During the course over two months of continuous operation both MFCs demonstrated efficient organics removal coupled with electrical power generation. However, higher maximal values of power output and COD removal were achieved by the JP bioreactor (Table 1). Also, bubbling on the top of anodic chambers of the JP bioreactor implied production of biogas. In order to observe community development and compare metabolic characteristics of microbial consortia from the bioreactors, whole community genomic DNA was isolated from the initial inoculums (UK-ref and JP-ref) and from anodic biofilms of MFCs 90 d and 70 d after bioreactor initiation (UK-mfc and JP-mfc, respectively). Shotgun sequence data were generated for each metagenomic library and further used for taxonomic and functional analysis (*Table S1*).

3.2 MFC microbial community taxonomic analysis

Metagenomic reads were pre-processed (Materials and Methods, *Figure S1*), and then assembled in contigs within each sample. Initially, biodiversity of the sample was estimated by 16S rRNA gene analysis (*Table S2*). Also, taxonomy was determined for each metagenomic sequence using the lowest common ancestor (LCA) approach (Figure 2**,** *Tables S3, S4, S5*). Interestingly, in spite of the numerous differences in the feed and operations of the two bioreactors, both approaches showed similar distributions of the major phylogenetic groups. The results obtained by the LCA method were used for quantitative estimation of population biodiversity.

The initial anaerobic sludge communities used for MFC inoculation differed by structure and diversity. The UK initial inoculum community (UK-ref) consisted of *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria*, with *Bacteroidetes* constituting over a half of the population. The JP initial inoculum (JP-ref) contained these same groups, with *Proteobacteria* predominant, but had a greater diversity of populations with a significant proportion of *Archaea* and some less represented bacterial phyla including *Chloroflexi*, *Thermotogae, Spirochaetes*, *Planctomycetes*, *Acidobacteria, Verrucomicrobia, Deinococcus-Thermus* and *Cyanobacteria* (Figure 2a,c).

Both initial communities underwent structural changes within 70-90 d of MFC operation. In the UK anode biofilm (UK-mfc), the proportions of *Proteobacteria*, *Firmicutes* and *Actinobacteria* increased compared to their abundance in the inoculum sludge. *Bacteroidetes,* although decreased in proportion to other bacterial groups, remained the most abundant phyla in the community. At the genus level, the most significant increase was shown for *Pseudomonas* and *Lactobacillus* (Figure 2b).

In the JP anode biofilm (JP-mfc), *Bacteroidetes, Firmicutes* and *Archaea* increased in abundance throughout MFC operation. *Proteobacteria* remained the most represented in the JP-mfc, although its fraction decreased compared to the corresponding initial community (Figure 2d,e). At the genus level, *Clostridium, Bacteroides, Prevotella, Pelobacter, Geobacter, Paludibacter* and *Methanothermobacter* were enriched in JP-mfc. Additional analysis of microbial DNA from different anodic components showed that the latter three genera and species related to *Roseiflexus* tend to accumulate on carbon cloth rather than on carbon granules (*Table S6*).

Thus, taxonomic analysis showed that *Bacteroidetes, Firmicutes* and *Proteobacteria* are present in distinct anaerobic sludge communities and dominant in MFCs fed by real industrial wastewater maintained under different environmental and operational conditions. The dominance and stability of these bacterial groups has previously been demonstrated in biogas reactors [33, 34] and in municipal wastewater-fed small-scale MFC anodic biofilms [16] whereas only *Bacteriodetes and Proteobacteria* but not *Firmicutes* were the dominant members of the metagenome of small-scale MFCs provided with acetate-rich feed [17]. Methanogenic *Archaea* were also well represented in JP-mfc (Figure 2d), consistent with the notable production of gas by this bioreactor.

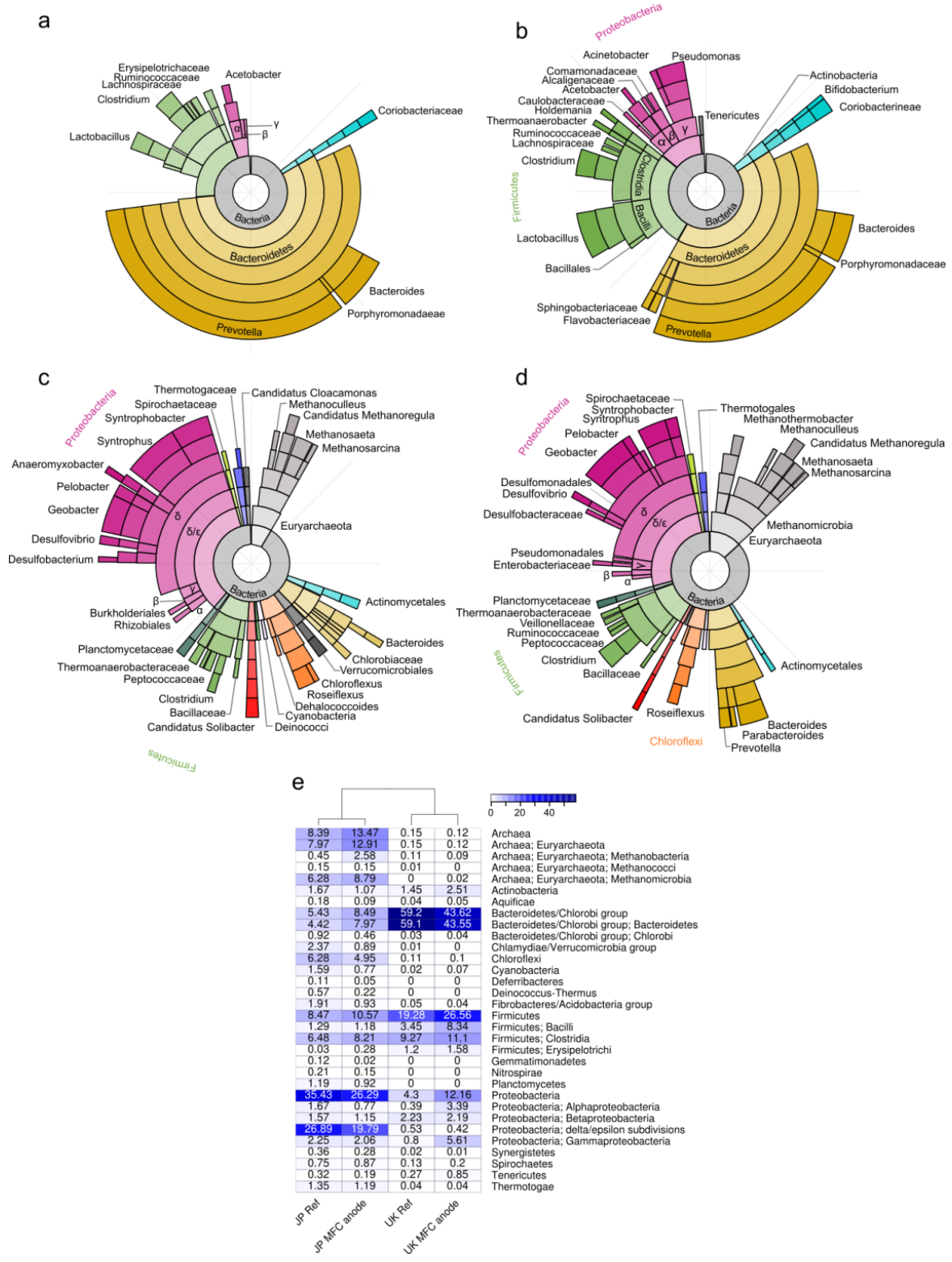

Figure 2: Phylogenetic profile of MFC microbiomes. Krone-like diagrams for UK ref (a), UK anode (b), JP ref (c), JP anode (d) represents the abundance of different phylogenetic groups within a sample where each circle represents taxonomic level, and colored according to the phylum level or group level (level 3 in NCBI taxonomy): yellow – *Bacteroidetes/Chlorobi* group, purple – *Proteobacteria*, light grey – *Archaea*, green – *Firmicutes*, orange – *Chloroflexi*, red – *Acidobacteria*, green-yellow – *Spirochaetes*, dark blue – *Thermotogae*, light blue – *Actinobacteria*, dark green – *Planctomycetes*, light pink – *Cyanobacteria*. (e) Heatmap representing the abundance of different phylogenetic groups on the level of phylum and class for most abundant groups; the numbers represent the percentage of abundance of a particular group in the sample.

The identified phylotypes along with well-known electricity-generating bacteria *Geobacter* spp. on anodic cloth are likely to contribute into direct electron transfer from the cell to anode surface. Among those identified, *Methanothermobacter* (*Archaea*) was earlier shown to increase power production in the mixed biofilms [35]. *Paludibacter* species were reported to be abundant in hydrogen producing microbial electrolysis cells [36], however, their impact on current generation has not yet been demonstrated. *Roseiflexus* species were previously found in the filamentous biofilm of cellulose-fed MFC, and their role in the community was associated with sugar utilization [37].

3.3 Most abundant molecular functions and genes responsible for bioremediation in MFC bioreactors

In microbial systems, taxonomic diversity is not necessarily related to functional diversity. Metagenome-based functional analysis of MFC communities was performed in order to examine their metabolic capacities. Besides general functional categories, we looked particularly into distribution of genes and pathways associated with bioremediation and extracellular electron transfer, two major functions of MFC communities.

Identified proteins were distributed among all existing Clusters of Orthologous Groups (COGs) functional categories [24] with "Carbohydrate transport and metabolism" and "Energy production and conversion" pathways overrepresented in the UK and JP bioreactor communities, accordingly **(**Figure 3a,b; *Table S7***)**. Linking identified functions to taxonomy showed that enriched pathways were introduced mostly by highly abundant phyla. In the UK bioreactor, the dominant group of *Bacteroidetes* was comprised of saccharolytic bacteria that derive energy primarily from carbohydrates and proteins through fermentation, and therefore hold a number of genes for carbon utilization. The enrichment of "Energy production and conversion" functional category in the JP-mfc was caused mostly by *Archaea* that added to methane metabolism pathways, as well as by *Proteobacteria* and *Firmicutes*.

Increased number of genes associated with motility, chemotaxis, signal transduction and membrane transport were found in the anodic biofilms compared to initial inoculums (*Table S8*). The most enriched proteins were critical for biofilm formation or important for energy utilization **(***Table S9***)**. Functional comparison between inoculum and anode communities showed gene enrichment in "Coenzyme transport and metabolism", "General function prediction only" and "Function unknown" categories on anode biofilms (*Table S7*) implying roles of yet unknown proteins in anodic biofilm formation and functioning.

The distribution of genes and pathways involved in biodegradation of the main components of distillery wastewaters, such as acetate, cellulose, starch and proteins was identified for anode biofilm communities. All examined pathways were introduced by higher number of microbial species in the more diverse JP-mfc populations (Figure 4**,** *Figures S2, S3*).

The main acetate-catabolizing enzymes (acetyl-CoA synthetase, phosphate acetyltransferase, acetate kinase, acetate permease, glyoxalate cycle enzymes) were mostly distributed between *Proteobacteria, Firmicutes* and *Archaea* (Figure 4). Distribution of key enzymes involved in anaerobic cellulose degradation (endogluconase, beta-glucosidase and exoglucanase) showed that *Firmicutes* were possibly the major cellulose degraders in both bioreactors (*Figure S2*). Starch was utilized mostly by abundant *Firmicutes, Bacteroidetes* and *Proteobacteria* in both MFCs, and also *Archaea* in JP-mfc (*Figure S3*).

Extracellular protein degradation can be performed by various proteases produced by different groups of microorganisms. An indirect way to determine the main contributors of proteolysis consists of searching for amino acid transporter genes in the community. Functional analysis showed that *Clostridia* might be the major bacterial family carrying out this function in both MFCs, with the help of *Lactobacillus* in the UK and *Delta-* and *Gammaproteobacteria* and *Archaea* in JP-mfc (data not shown).

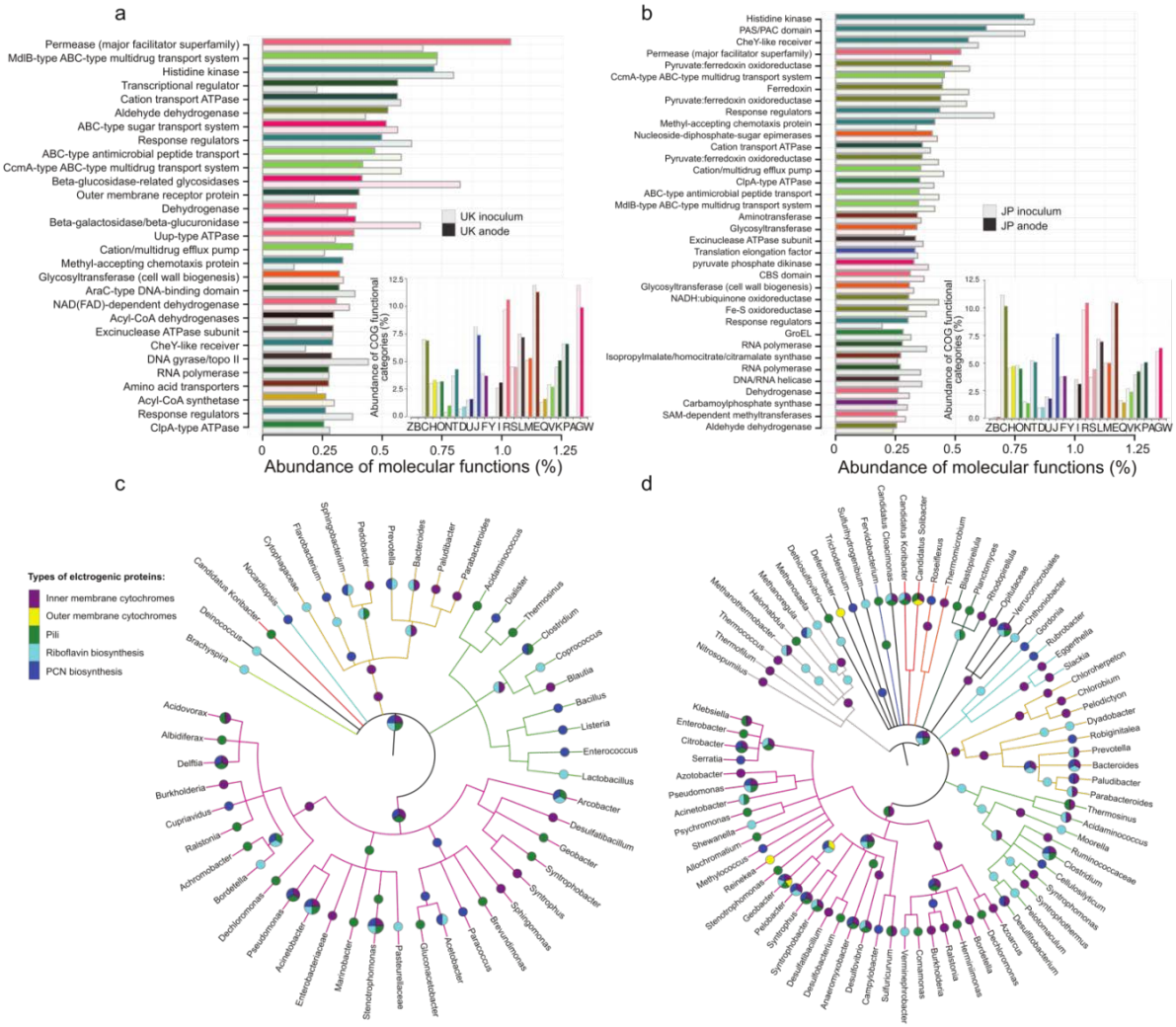

Figure 3: Functional profile of MFC microbiomes, and distribution of different types of proteins involved in electron transduction to anode among different phylogenetic groups on anodic biofilm. (a,b) Representation of the most abundant orthologous groups in the anode biofilm, represented by at least by 0.25% in (a) UK MFC or (b) JP MFC as determined by assignment to the COG database by MG-RAST. Orthologous groups are colored by their representative functional category, translucent bands represent the abundance in corresponding reference metagenomes. (c,d) Distribution of homologous of known proteins involved in extracellular electron transfer determined by BLAST (similarity 45%, E-value 10^{-5}, alignment length at least 60 aa) plotted on NCBI taxonomy tree for (c) UK MFC and (d) JP MFC. Branches are colored according to the phylum level, circles on the branches indicate the presence of particular type of proteins in this phylogenetic group. For detailed information see Table S10. A key to the COG letter codes for functional categories can be found in Table S7, a detailed description for particular COG can be found in Table S8.

3.4 Diversity of genes related to extracellular electron transfer in the anodic biofilm communities

Our knowledge on extracellular electron transfer mechanisms is mostly built on recent studies of two genera of dissimilatory metal-reducing bacteria, *Geobacter* and *Shewanella*. These bacteria use an assembly of membrane and periplasmic *c*-type cytochromes to move catabolic electrons across the cell envelope directly to extracellular solid conductive surfaces [32]. In addition, extracellular electron transfer via pili-like microbial nanowires was clearly

demonstrated for *Geobacter* species [38, 39] which use type IV pili composed of PilA for conduction of electrons through multilayer biofilms [40]. In *Shewanella* direct electron transfer can occur through outer membrane protein extensions [10] or via soluble electron shuttles produced by cells [41, 42].

Metagenome-wide screening of anode biofilm sequences showed the presence of the key genes for both direct and indirect extracellular electron transfer types in both bioreactors. However, the distribution of these genes and their taxonomic assignment differed between the two MFCs (Figure 3c,d; *Table S10*).

Sequences similar to the genes encoding *c*-type cytochromes of the "metal respiration" system were found in both biofilms and mostly belonged to different families of *Deltaproteobacteria* or *Gammaproteobacteria* in the JP-mfc and UK-mfc, respectively. Notably, outer membrane (OmcABESZ, MtrF) and periplasmic (PpcA, MtrA/D) *c*-type cytochromes required for electron exchange between the cell surface and the anode were found only in the JP-mfc among *Geobacter*, *Deferribacter*, *Reinekea* and some unidentified species. The sequences similar to the inner membrane *c*-type cytochromes (MacA, CymA, SirC) were found in both bioreactors, however with significantly higher and taxonomically wider representation in the JP-mfc. The difference in the distribution of electrode respiration related *c*-type cytochromes between the MFCs can be explained by a higher amount of metal-reducing *Deltaproteobacteria* in the JP-mfc.

Among taxonomically identified sequences most of pili formation genes were possessed by *Proteobacteria*, especially by *Delta-* and *Gamma-* classes in JP-mfc and UK-mfc, accordingly. The JP-mfc but not the UK-mfc possessed *Geobacter* PilA homologs. The mannose-sensitive hemagglutinin (Msh) pili, have been shown to be necessary for optimal current generation in *Shewanella* [43]. The Msh pili sequences showed broader variation in taxonomy, particularly in the JP-mfc, where *msh* genes were assigned to *Firmicutes*, *Planctomycetes*, *Verrucomicrobia*, *Thermotogae*, *Acidobacteria*, different classes of *Proteobacteria* and *Archaea*. In the UK-mfc, genes similar to *msh* were found in *Firmicutes* and all classes of *Proteobacteria*. Interestingly, all *msh* fragments identified in *Epsilonproteobacteria* were most similar to *Arcobacter butzlery*, an electrogenic bacterium previously isolated from similarly configured MFC bioreactor [44].

Undirected extracellular electron transfer is carried out via electron shuttles produced by some bacteria. It has been suggested that bacterial shuttle secretion could be stimulated by current generation in MFCs [45]. The endogenously secreted flavins of *Shewanella* species, mainly riboflavin, are the most well documented electron shuttles in MFCs [46, 47, 48]. Also, it is known that phenazine-based metabolites produced by *Pseudomonas* species can function as electron shuttles for *Pseudomonas* cells themselves and also, in a syntrophic association, for Gram-positive bacteria [49].

The *ribB* gene, which encodes a riboflavin synthesis enzyme, and most genes involved in the phenazine-1-carboxamide synthesis pathway (*phzDEFG*) were detected in both biofilms and were related mainly to *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. The electron shuttle gene sequences had broader taxonomic distribution in the JP-mfc, where fragments belonging to *Deltaproteobacteria*, *Verrucomicrobia* and *Archaea* were found. *Alphaproteobacteria* shuttle sequences were found only in the UK-mfc.

Based on the abundance of extracellular transfer genes in the two MFC anode biofilms, electrochemical activity involved both direct and mediated electron transfer in the JP MFC, and was mainly due to excreted redox components in the UK bioreactor.

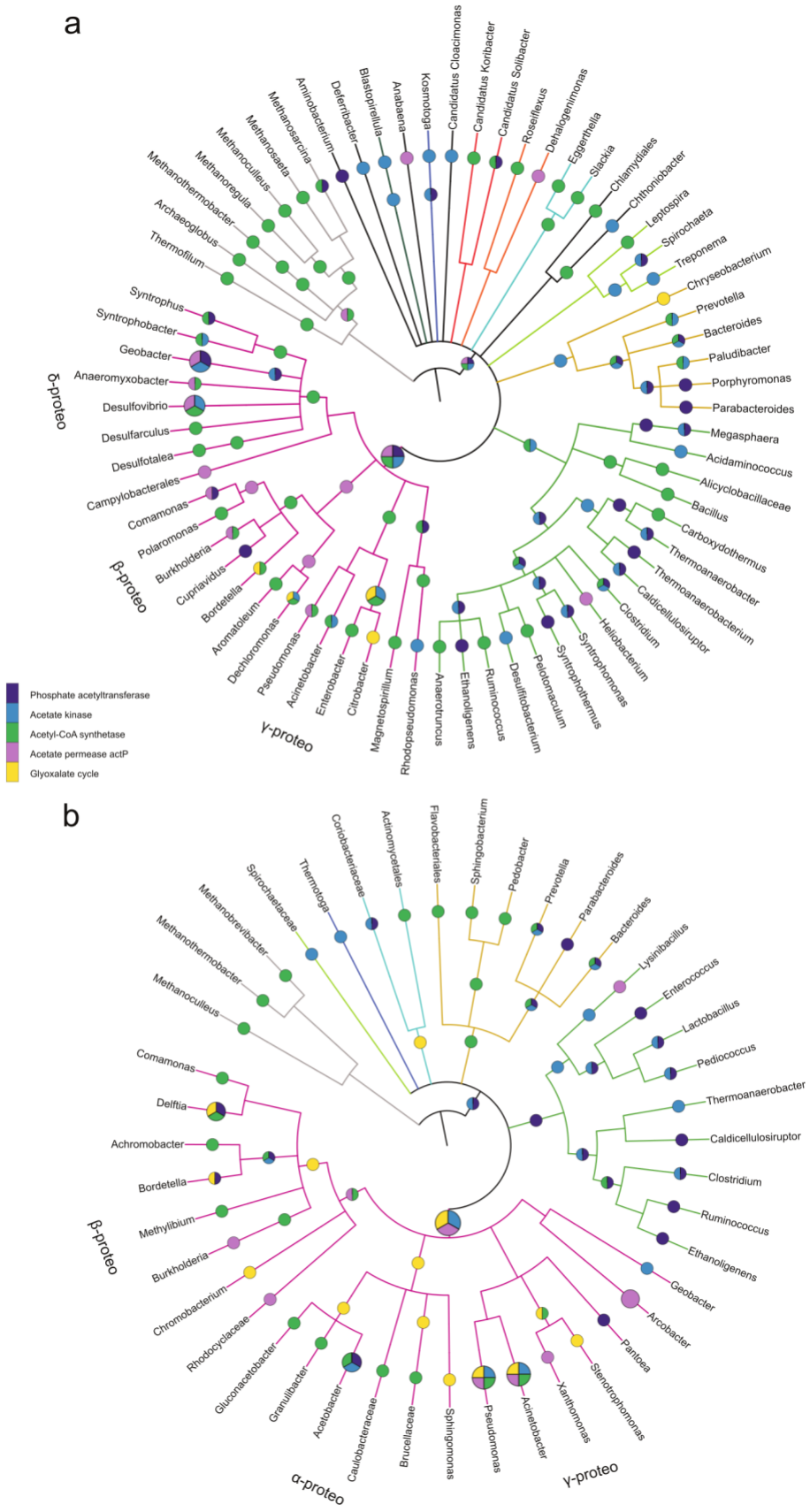

Figure 4: Acetate utilization by anodic microbial communities. Distribution of the BLAST hits (similarity 45%, E-value 10^{-5} and alignment length at least 60 aa) and HMMer search for enzymes involved in acetate utilization against metagenome protein database plotted on NCBI taxonomy tree for (a) JP anode or (b) UK anode. Branches are colored according to the phylum level, circles indicate the presence of the specified enzymes in the particular phylogenetic group.

4 Concluding remarks

Identification of key microorganisms that drive bio-electroremediation is the first step for determining the most effective biocatalyst for wastewater-treating MFC systems. Our study whole-genome metagenomic study was conducted on large volume (60-64 L) MFCs operating under conditions that would be expected for real-world treatment in a modular MFC system. The present analysis showed that three bacterial phyla, *Proteobacteria*, *Firmicutes* and *Bacteroidetes* constituted the core of electrochemically active biofilms in independently-inoculated MFCs fed with different industrial wastewaters in distinct climatic zones.

The microbial community of the MFC operated in the subtropical climate (Okinawa, Japan) had higher biodiversity and held greater gene repertoire for biodegradation and extracellular electron transfer compared to temperate climate population (Edinburgh, UK). The JP MFC was dismantled following the 70 d experimental period reported here but the UK MFC continued to maintain stable electrical output until its decommissioning approximately one year later (unpublished results), demonstrating the resilience of its electrogenic community. The observation of biogas production from the JP MFC implies a possibility for integrated organics removal, electrical power generation and biogas production from one functionally diverse microbial community.

Based on the results of the present study, the MFC bioreactor operating in Edinburgh could potentially have benefited from enrichment with exoelectrogenic microorganisms that use direct electron transfer mechanisms, such as *Geobacter* spp. Also, providing conditions favorable for methanogenic microbes could enhance organics removal without a significant loss in electrical power production.

Availability of the metagenomic data of mixed anaerobic sludge and electricity generating biofilms will further allow for targeted studies of the enzymes involved in bio-electroremediation and should eventually lead to breakthroughs in metabolic modeling and our ability to predict, develop and maintain functionally stable and robust electrogenic microbial communities optimized for specific waste treatment in a particular geographical location.

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