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1 **Short Communication**

Use of SWATH mass spectrometry for quantitative proteomic investigation of *Shewanella oneidensis* MR-1 biofilms grown on graphite cloth electrodes

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1 **Abstract**

2 Quantitative proteomics from low biomass, biofilm samples is not well documented. In this
3 study we show successful use of SWATH-MS for quantitative proteomic analysis of a
4 microbial electrochemically active biofilm. *Shewanella oneidensis* MR-1 was grown on
5 carbon cloth electrodes under continuous anodic electrochemical polarizations in a
6 bioelectrochemical system (BES). Using lactate as the electron donor, anodes serving as
7 terminal microbial electron acceptors were operated at three different electrode potentials
8 (+0.71V, +0.21V & -0.19V vs. SHE) and the development of catalytic activity was monitored
9 by measuring the current traces over time. Once maximum current was reached (usually
10 within 21-29 hours) the electrochemical systems were shut off and biofilm proteins were
11 extracted from the electrodes for proteomic assessment. SWATH-MS analysis identified 704
12 proteins, and quantitative comparison was made of those associated with tricarboxcylic acid
13 (TCA) cycle. Metabolic differences detected between the biofilms suggested a branching of
14 the *S. oneidensis* TCA cycle when grown at the different electrode potentials. In addition, the
15 higher abundance of enzymes involved in the TCA cycle at higher potential indicates an
16 increase in metabolic activity, which is expected given the assumed higher energy gains. This
17 study demonstrates high numbers of identifications on BES biofilm samples can be achieved
18 in comparison to what is currently reported. This is most likely due to the minimal
19 preparation steps required for SWATH-MS.

20 **Abbreviations:** BES, Bioelectrochemical systems; EET, Extracellular electron transfer;
21 TCA, Tricarboxylic Acid; IDA, Information Dependent Acquisition; NADP, Nicotinamide
22 Adenine Dinucleotide Phosphate; NAD, Nicotinamide Adenine Dinucleotide; SHE, Standard
23 Hydrogen Electrode; KCl, Potassium Chloride; NCBI, National Center for Biotechnology
24 Information

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1 **Keywords:** Bioelectrochemical system; electroactive biofilms; Proteomics; *Shewanella*
2 *oneidensis* MR1; SWATH-MS

3 **Introduction**

4 Bioelectrochemical systems (BESs) exploit microbial oxidation and reduction reactions to
5 catalyse extracellular electron transfer (EET) and cause electron flow between an anode and a
6 cathode [22]. Currently, there is great interest around EET research, as BESs have many
7 potential applications, including bioremediation and production of valuable chemicals and
8 fuels [22]. The more common microorganisms found to proliferate in BES anodic
9 compartments are dissimilatory metal reducing bacteria [11]. For an oxidation process at an
10 anode, dissimilatory metal reducing bacteria harness the energy generated through the
11 oxidation of simple substrates (e.g. fatty acids) and the transfer of electrons to the anode
12 electrode [19]. Here they form biofilms on anodes that perform EET and provide the current
13 to the BES [23].

14 *Shewanella* and *Geobacter* species are the most intensively studied bacteria for extracellular
15 respiration in BES. Both are known to respire using solid electron acceptors by passing
16 electrons through redox-proteins dominated by c-type cytochromes for EET [27]. *Shewanella*
17 *oneidensis MR-1* is extremely versatile in that it has the ability to reduce a wide array of
18 electron acceptors [18, 30]. It has 42 possible c-type cytochromes [16] and it is apparent that
19 alternative cytochromes are used within its respiration pathways, indicating modularity of the
20 electron transfer mechanisms of *S. oneidensis* [7]. There is much interest in the electron
21 transfer mechanisms of *S. oneidensis* with a recent study proposing that nanowires are
22 extensions of the outer membrane and periplasim and containing key cytochromes involved
23 for EET [21]. There have been several proteogenomic studies conducted on *Shewanella* [8, 9,
24 14, 24]. However, these studies focus specifically on proteomics with the aim to improve

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1 annotation of the genome and are not comparative in that they do not study *Shewanella* under
2 different conditions.

3 Within the scope of BES, many studies to date combine electrochemical aspects, e.g. growing
4 active biofilm and optimizing current production, with microbial physiology. A recent study
5 demonstrated biofilms of *Shewanella putrefaciens* produced more current and more biofilm
6 with increasing anode potential [2]. Other studies also reveal a strong effect of the anode
7 potential on EET [10, 15]. Consequently, there is great interest to study the proteomic basis
8 of the adaptation of the model organism *Shewanella oneidensis* MR-1 to different electrode
9 potentials.

10 Recent molecular investigations of BES biofilms have aimed to determine the mechanistic
11 details of the EET process [4, 13, 17, 25]. For example, examination of *S. oneidensis* gene
12 expression discovered that up-regulation of genes coding for certain respiratory proteins
13 (*mtrABC*, *omA* and *cctA*) occurred when using an electrode as opposed to oxygen or iron
14 citrate as the terminal electron acceptor [25]. Recently, the first quantitative proteomic study
15 was performed to determine details of EET [20]. Protein abundances of *Arcobacter butzleri*
16 *ED-1* using either oxygen or a BES electrode as the terminal electron acceptor were
17 compared [20]. Notable findings were that two novel cytochromes, potentially involved in
18 EET, and flagellin were upregulated during growth on the electrode. These initial yet
19 pioneering studies applying 'omic (including genomic, proteomic, transcriptomic or
20 metabolomic) approaches are providing unique insight into the mechanisms of EET.

21 Further application of proteomics could be utilised to reveal metabolic and physiological
22 details of the microorganisms performing EET. However, BESs are often operated at a small
23 scale for convenience and to simplify operation. This becomes problematic for proteomic
24 studies that require enough biomass for adequate protein extraction, especially for

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1 quantitative analyses. Indeed it was apparent that cell biomass levels were a problem in the
2 *Arcobacter* study mentioned above [20], as replicate electrode samples were pooled for
3 quantitative iTRAQ analysis, and relatively low numbers of unique proteins were detected,
4 ranging from 115 to 233, from any particular sample. Consequently, these shortfalls limit the
5 outcomes of proteomic investigations of electroactive biofilms in BES.

6 SWATH-MS is a recently developed approach that provides extensive label-free quantitation
7 of the measurable peptide ions in a sample [33]. The approach rapidly acquires high
8 resolution Q-TOF mass spectrometer data through repeated analysis of sequential isolation
9 windows (swaths) throughout the chromatographic elution range [6]. Of the few reports on
10 the use of SWATH-MS for bacterial proteomics, to our knowledge this is the first to use the
11 method on low biomass electrode biofilms.

12 Here we compared the proteome of *S. oneidensis* to detect functional differences while
13 growing on an anodic electrode at different potentials. We show successful quantitative
14 proteomic analysis of the anodic *Shewanella* biofilm samples using SWATH-MS without the
15 need for fractionation, labelling or other procedures that can contribute to protein losses.
16 Furthermore, as a result of the high numbers of identifications and quantitative data obtained
17 from this study, we propose this procedure is very well suited for proteomic studies of low
18 biomass biofilms.

19

20 **Materials and Methods**

21 Three sterile BESs were assembled under anaerobic conditions and filled with a defined
22 minimal medium for all experiments using 18mM of lactate as the electron donor, carbon
23 cloth as the electron acceptor and titanium wire as the counter electrode (refer to

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1 Supplementary Information for details of BESs operation). Each BES was inoculated with *S.*
2 *oneidensis* at a constant OD_{600}. All experiments were conducted in triplicate anaerobically
3 under potentiostatic control using the Ag/AgCl reference electrode (3M KCl, BASi (USA)) at
4 30°C. *S. oneidensis* biofilms were grown at the anodes of bioelectrochemical reactors
5 polarised at +0.71, +0.21 and -0.19 V vs SHE. These batch chronoaperometric experiments
6 continued until near maximum currents were produced (Figure 1A). Anodic electrodes were
7 then removed from replicate BES reactors under anaerobic conditions and stored at -80°C.
8 Used medium was filtered and analysed for lactate and acetate concentration using HPLC
9 (see the Supplementary Information for details).

10 Protein extractions were performed to maximise protein yield from the whole electrodes
11 containing biofilms. Precise details are described in the Supplementary Information. In brief,
12 electrodes were submerged in extraction buffer and subjected to three freeze/thaw cycles and
13 sonicated to further lyse cells. The electrode was rinsed with additional extraction buffer
14 which was combined with the cell lysate. The extraction solutions were centrifuged to
15 remove cell debris and proteins were then precipitated. Protein was recovered through
16 centrifugation, washed in cold acetone and resuspended in buffer. Total resuspended protein
17 was quantified then reduced, alkylated and digested.

18 Following purification, 1µg from each digested protein sample was used for SWATH-MS
19 analyses. Additional 2µg aliquots of each sample were pooled in duplicate to create a spectral
20 library using IDA mode mass spectrometry. Samples were analysed using a Triple-Tof5 600
21 instrument (ABSciex). Mass spectrometry (MS) data from IDA analyses were combined and
22 searched using ProteinPilot software v4.5 (ABSciex, Forster City CA) a database of
23 *Shewanella oneidensis* MR-1 specific proteins acquired from NCBI on the 28th May 2012
24 containing 4052 entries. A decoy database containing reversed protein sequences was used to

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1 determine the false discovery rate of identifications. The IDA spectral library, protein
2 sequences and SWATH MS data were loaded into PeakView software v1.2 for processing.
3 The MSstats program [3] was used for statistical analysis of the acquired spectra and Pathway
4 Tools [12] was used to visualise the statistical data on metabolic pathways of *S. oneidensis.*
5

6 **Results and Discussion**

7 During operation of the BES, current production by *S. oneidensis* in the BES increased over
8 time for all the anodic potentials of +0.71V, +0.21V and -0.19V (SHE) (Figure 1A). Higher
9 current densities were achieved at anodes poised at higher potentials. The amounts of protein
10 extracted from the electrode biofilms were consistent between replicates, with higher
11 amounts obtained from the electrodes at higher potentials (Figure 1B).

12 A total of 740 unique proteins were identified within the library acquired by information
13 dependant acquisition (IDA) with a false detection rate of 0.01 calculated using a Paragon
14 method within the ProteinPilot software. Of these unique proteins SWATH-MS analysis
15 detected 704 in each biofilm sample. The number of significantly different ($p<0.05$)
16 abundant proteins was determined between pairwise comparisons of the BES biofilms
17 developed at the different potentials. There were 58, 115 and 41 differentially abundant
18 proteins between the comparisons of +0.21 V to - 0.19 V, +0.71 V to -0.19 V and +0.71 V to
19 +0.21 V respectively ($\log_2 FC>1$, $p<0.05$).

20 The greatest number of significantly different abundant proteins was between electrode
21 biofilms at the potentials of +0.71 and -0.19 V. The TCA cycle is an essential metabolic
22 pathway enabling energy generation and synthesis for many microorganisms. Consequently,
23 to demonstrate detection of metabolic differences we focused on comparison of proteins

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1 involved in the bacterial TCA cycle at these electrode potentials (Figure 2). Although the
2 TCA cycle typically operates under aerobic conditions, *S. oneidensis* has been shown to use
3 this pathway partially during anaerobic respiration coupled to alternative electron acceptors
4 such as fumarate and TMAO [1, 28].

5 The $\log_2$ fold change ($\log_2$FC) for the majority of the TCA cycle proteins in the comparison
6 of the +0.71 V to - 0.19 V anode biofilms were positive (Figure 2). This indicated a higher
7 abundance of these proteins at +0.71 V and a more active TCA cycle in comparison to -0.19
8 V. This correlates with the BES chronoamperometry results, with +0.71 V showing
9 significantly higher current production and thus overall metabolic activity than that detected
10 at – 0.19 V (Figure 1A). This higher electron transfer rate was generated through increased
11 carbon substrate (lactate) oxidation activity at the higher anode potential. The enzymes
12 involved in the conversion of lactate to pyruvate (Dld, LldE,LldF & LldG) were higher in
13 abundance at +0.71 V ($p<0.05$), suggesting a higher rate of carbon metabolism at the higher
14 potential. This activity was confirmed as lactate utilisation was higher in the +0.71 V culture
15 in comparison to the -0.19 V culture (SI Table 1).

16

17 Proteins of the TCA cycle with negative $\log_2$FC were relatively in higher abundance at -0.19
18 V. The protein MaeB was statistically more abundant at -0.19 V and this enzyme catalyses an
19 NADP- dependent conversion of malate to pyruvate (Figure 2). The protein MaeA, a NAD-
20 dependent malic enzyme was more abundant at +0.71 V. This protein carries out the same
21 reaction, however this uses NAD^+ rather than $NADP^+$ for conversion of malate to pyruvate. In
22 general bacterial metabolism, conversions utilising the NAD^+/NADH couple are involved in
23 oxidative catabolic reactions and respiratory electron transfer [5]. In contrast the
24 NADP+/NADPH couple is utilised in anabolic reactions [5]. This appears to be a response of

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1 the cells corresponding to the different electrode potentials and is in agreement with the
2 outcomes observed here in that more respiratory activity (NAD+ reactions) was evident at
3 +0.71 V compared to -0.19 V (Figure 1A). The higher potential of the anode would provide
4 more opportunity for electron transfer through the respiratory pathway, given the higher
5 energy gain associated with electron transfer between redox couples at greater potential
6 difference.

7

8 Conversely, at low potential it is possible that the TCA cycle is functioning at a decreased
9 level. Although under more reduced conditions, NADH levels will be high and this is known
10 to inhibit key oxidative enzymes in the cycle [32]. Several studies report that under anaerobic
11 conditions *S. oneidensis* possesses an incomplete TCA cycle [26], using either an oxidative or
12 reductive branch for production of cell intermediates [1, 28]. However, activity of a complete
13 TCA cycle has been detected under certain anaerobic conditions [28]. Although, in that
14 instance the carbon flux through the TCA cycle was very low and acetate was a major
15 product of lactate oxidation [28]. That was not the case in this study at the higher potential, as
16 acetate production was less than 5% of the consumed lactate (Table S1), this result
17 supporting the scheme of lactate utilisation proceeding through the TCA cycle. Conversely,
18 acetate production at the low potential was significant (Table S1), and this activity has been
19 observed previously in anaerobic conditions [1]. Consequently, at the low potential the
20 acetate production was important for substrate level ATP production.

21

22 The reactions of the TCA pathway that are utilised would have great impact on the number of
23 electrons consumed/produced [18], and the choice of those used is likely a dynamic process

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1 determined by environmental conditions [1]. When looking at protein abundances for each
2 side of the TCA cycle, there is evidence to support this suggestion. At +0.71 V we see higher
3 abundances for proteins involved in energy generating reactions, suggesting that the complete
4 TCA cycle is being extensively utilised. Conversely at -0.19V we observed equal abundances
5 for proteins involved in the reductive branch of the cycle, suggesting in these conditions there
6 is less use of the oxidative branch of the TCA cycle. This is in agreement with previous
7 observation where *S. oneidensis* uses a complete TCA cycle at higher redox potential and
8 utilisation of the branched cycle was evident at lower redox potential [28]. With regard to the
9 TCA cycle, the proteomic findings made here are in agreement with what is expected from
10 the metabolic and energetic activities of *S. oneidensis*.

11 The number of protein identifications achieved in this study improves on quantitative
12 proteomic investigations of an electrode biofilm. The SWATH-MS approach used here is
13 advantageous for proteomic analysis on samples where biomass or protein quantities are very
14 low. The sensitivity of SWATH-MS removes the need for fractionation and being label free,
15 removes the need for several processing steps involved with labelling procedures which may
16 contribute to loss of protein [29]. The extraction method in combination with IDA analysis
17 successfully obtained high levels of identifications from the electrode attached biofilm
18 samples. In particular, this method could be used for detailed interrogation of the electron
19 transfer proteins of BES biofilms.

20 Microbial electrochemical systems like microbial fuel cells have attracted attention as a
21 promising alternative to unsustainable energy sources and technologies. Among the
22 development of other components, the improved understanding and details of EET pathways
23 of model organisms, such as *S. oneidensis MR-1*, provides opportunity to fine tune reactor
24 conditions to the metabolic capabilities of the organism and achieve improved process

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1 performance. Establishing the SWATH-MS approach in this field opens the way for further
2 investigations to improve our understanding of electroactive biofilms for advancing the BES
3 technology

4 The SWATH-MS analysis is quantitative and enabled a relative comparison of protein
5 abundance between our biofilm samples. Using this technique we gained evidence that the
6 TCA cycle of *S. oneidensis* electrode biofilm is more active when grown at a higher potential
7 (+0.71 V). The results also suggest that at lower potential, utilisation of reactions dependent
8 on NADPH rather than NADH was preferred, and this likely reflects decreased respiratory
9 activity in this condition. Consequently, we suggest the use of the above mentioned
10 extraction and SWATH-MS for quantitative proteomic analysis of electrode biofilm samples,
11 and in general from samples where the quantity of protein is limited.

12

13 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
14 Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository
15 [31] with the dataset identifier PXD001472.

16

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The authors have declared no conflict of interest.

Figure 1: Profiles of current production from *Shewanella oneidensis MR-1* at different anode potentials within the BES over time (A). Amounts of protein extracted from electrode biofilms of *S. oneidensis* after BES operation at different anode potentials (error bars indicate standard deviation) (B).

Figure 2: *Shewanella oneidensis* MR-1 prokaryotic TCA cycle as adapted using Pathway Tools software. The colour coded expression ratios indicate the Log2 Fold Change occurring between protein abundances in +0.71V relative to -0.19V electrode biofilms. Full names of the abbreviated proteins are located in Table S2. Inset box shows the abundance differences between the multiple enzymes that carry out the conversion of lactate to pyruvate. All Log2 Fold Change values are significant ($p<0.05$) unless indicated with an asterisk (*).

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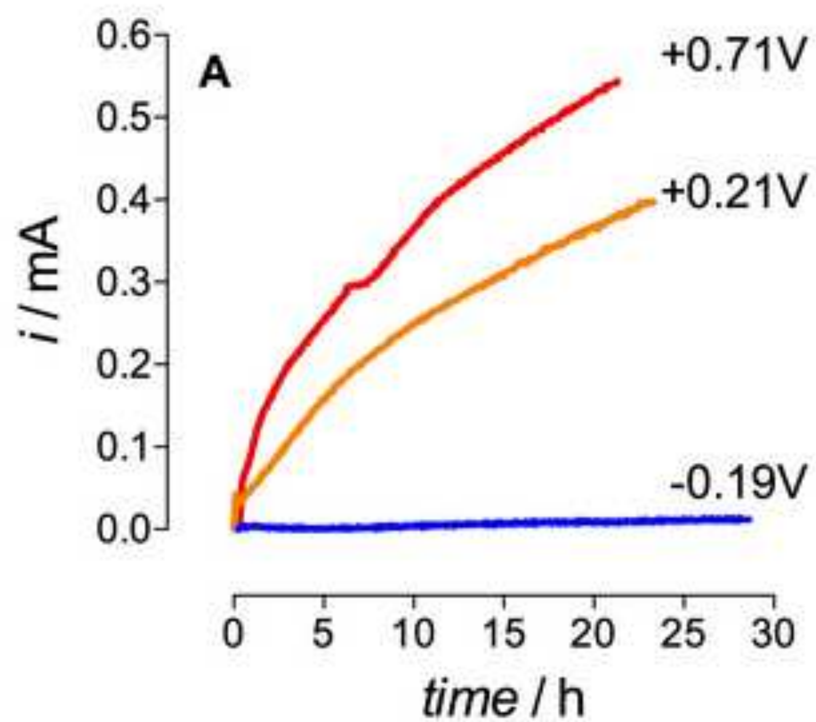
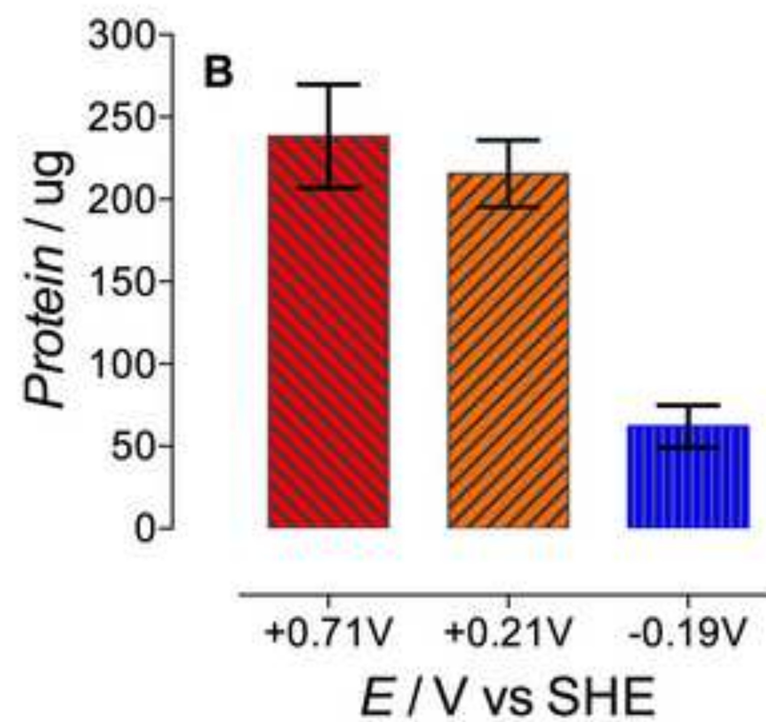
Figure 1





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